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Agency

Health Effects Support Document for Cyanobacterial Toxins: microcystins, anatoxin-a, and cylindrospermopsin

Health Effects Support Document For Cyanobacterial Toxins: microcystins, anatoxin-a, and cylindrospermopsin

U.S. Environmental Protection Agency
Office of Water (4304T)
Health and Ecological Criteria Division
Washington, DC 20460

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FOREWORD

The Safe Drinking Water Act (SDWA), as amended in 1996, requires the Administrator of the U.S. Environmental Protection Agency (U.S. EPA) to establish a list of unregulated microbiological and chemical contaminants that are known or anticipated to occur in public water systems and that may need to be controlled with a national primary drinking water regulation. The SDWA also requires that the Agency make regulatory determinations on at least five contaminants on the list every five years. For each contaminant on the CCL, the Agency will need to obtain sufficient data to conduct analyses on the extent of occurrence and the risk posed to populations via drinking water. Ultimately, this information will assist the Agency in determining the appropriate course of action (e.g., develop a regulation, develop guidance, or make a decision not to regulate the contaminant in drinking water).

This document provides the health effects basis for the development of Drinking Water Health Advisories (DWHA) for the cyanobacterial toxins microcystins, anatoxin-a, and cylindrospermopsin. DWHA serve as the informal technical guidance for unregulated drinking water contaminants to assist Federal, State and local officials, and managers of public or community water systems in protecting public health as needed. They are not to be construed as legally enforceable Federal standards. In order to avoid wasteful duplication of effort, information from the following risk assessments were used in the development of this document.

- Health Canada (2012) Toxicity Profile for Cyanobacterial Toxins
- Enzo Funari and Emanuela Testai (2008) Human Health Risk Assessment Related to Cyanotoxins Exposure
- Tai Nguyen Duy, Paul Lam, Glen Shaw and Des Connell (2000) Toxicology and Risk Assessment of Freshwater Cyanobacterial (Blue-Green Algal) Toxins in Water
- National Institute of Environmental Health Sciences (2000) Cylindrospermopsin [CASRN 143545-90-8] Review of Toxicological Literature

A Reference Dose (RfD) is provided as the assessment of long-term toxic effects other than carcinogenicity. RfD determination assumes that thresholds exist for certain toxic effects, such as cellular necrosis, significant body or organ weight changes, blood disorders, etc. It is expressed in terms of milligrams per kilogram per day (mg/kg/day) or micrograms per kilogram per day (µg/kg/day). In general, the RfD is an estimate (with uncertainty spanning perhaps an order of magnitude) of a daily oral exposure to the human population (including sensitive subgroups) that is likely to be without an appreciable risk of deleterious effects during a lifetime.

The carcinogenicity assessment for microcystins, anatoxin-a, and cylindrospermopsin includes a formal hazard identification and an estimate of tumorigenic potency if applicable. Hazard identification is a weight-of-evidence judgment of the likelihood that the agent is a human carcinogen via the oral route and of the conditions under which the carcinogenic effects may be expressed.

Development of these hazard identification and dose-response assessments for microcystins, anatoxin-a, and cylindrospermopsin has followed the general guidelines for risk assessment as set forth by the National Research Council (1983). U.S. EPA guidelines that were used in the development of this assessment include the following:

- *Guidelines for the Health Risk Assessment of Chemical Mixtures* (U.S. EPA, 1986a)
- *Guidelines for Mutagenicity Risk Assessment* (U.S. EPA, 1986b)
- *Recommendations for and Documentation of Biological Values for Use in Risk Assessment* (U.S. EPA, 1988)
- *Guidelines for Developmental Toxicity Risk Assessment* (U.S. EPA, 1991)
- *Interim Policy for Particle Size and Limit Concentration Issues in Inhalation Toxicity* (U.S. EPA, 1994a)
- *Methods for Derivation of Inhalation Reference Concentrations and Application of Inhalation Dosimetry* (U.S. EPA, 1994b)
- *Use of the Benchmark Dose Approach in Health Risk Assessment* (U.S. EPA, 1995)
- *Guidelines for Reproductive Toxicity Risk Assessment* (U.S. EPA, 1996)
- *Guidelines for Neurotoxicity Risk Assessment* (U.S. EPA, 1998)
- *Science Policy Council Handbook*: *Risk Characterization* (U.S. EPA, 2000a)
- *Benchmark Dose Technical Guidance Document* (U.S. EPA, 2000b)
- *Supplementary Guidance for Conducting Health Risk Assessment of Chemical Mixtures* (U.S. EPA, 2000c)
- *A Review of the Reference Dose and Reference Concentration Processes* (U.S. EPA, 2002)
- *Guidelines for Carcinogen Risk Assessment* (U.S. EPA, 2005a)
- *Supplemental Guidance for Assessing Susceptibility from Early-Life Exposure to Carcinogens* (U.S. EPA., 2005b)
- *Science Policy Council Handbook: Peer Review* (U.S. EPA, 2006a)
- *A Framework for Assessing Health Risks of Environmental Exposures to Children* (U.S. EPA, 2006b)

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ABBREVIATIONS AND ACRONYMS

Adda	3-amino-9-methoxy-2, 6, 8,-trimethyl-10-phenyldeca-4, 6-dienoic acid
ADHD	Attention deficit hyperactivity disorders
ADI	Acceptable Daily Intake
ALDH2	Aldehyde dehydrogenase 2
ALT	Alanine aminotransferase
ALP	Alkaline phosphatase
ANTX	Anatoxin-a
APHA	American Public Health Association
AST	Aspartate aminotransferase
AWWARF	American Water Works Association Research Foundation
BGAS	Bluegreen algae supplements
BMD	Benchmark dose
BMDL	Benchmark dose level
BMDS	Benchmark dose software
BMR	Benchmark response
BSO	Buthionine sulfoximine
BW	Body weight
CAS	Chemical Abstracts Service
CEGHLL	Center of Excellence for Great Lakes and Human Health
CCL	Contaminant Candidate List
CDC	Centers for Disease Control and Prevention
CHO	Chinese hamster ovary
CI	Confidence Interval
CTA	Cell transformation assay
CYN	Cylindrospermopsin
DAF	Dose adjustment factor
DMBA	Dimethylbenzanthracene
DNA	Deoxyribonucleic acid
DWHA	Drinking Water Health Advisories
ELISA	Enzyme-linked immunosorbent assay
EPA	U.S. Environmental Protection Agency
FDA	U.S. Food and Drug Administration
FEL	Frank effect level
GD	Gestation day
GC/MS	Gas chromatograph/mass spectrometry
GFR	Glomerular filtration rate
GGT	y-Glutamyl transpeptidase
GI	Gastrointestinal

GIS	Geographical information system
GSH	Glutathione
GST	Glutathione S-transferase
HA	Health advisory
HAB	Harmful algal bloom
HCT	Hematocrit
HED	Health effect dose
HESD	Health effect support document
HPLC	High-performance liquid chromatography
IARC	International Agency for Research on Cancer
IgE	Immunoglobulin E
i.p.	Intraperitoneal
LC/MS	Liquid chromatography/mass spectrometry
LDH	Lactate dehydrogenase
LOAEL	Lowest-observed-adverse-effect level
LPS	Lipopolysaccharides
MC-LA	Microcystin-LA
MC-LR	Microcystin-LR
MC-RR	Microcystin-RR
MC-YR	Microcystin-YR
MC-YM	Microcystin-YM
MCL	Maximum Contaminant Level
MCLG	Maximum Contaminant Level Goal
MDA	Malondialdehyde
Mdha	Methyldehydroalanine
MDNR	Maryland Department of Natural Resources
MDPH	Massachusetts Department of Public Health
MERHAB-LGL	Monitoring and Event Response to Harmful Algal Blooms in the Lower Great Lakes
MMP	Metalloproteinase
MN	Micronuclei
MNBNC	Micronucleated binucleate cells
MOA	Mode of action
MPT	Mitochondrial permeability transition
mRNA	Messenger RNA
MW	Molecular weight
MWDSC	Metropolitan Water District of Southern California
N/A	Not Applicable
NDEA	N-nitrosodiethylamine
NHDES	New Hampshire Department of Environmental Services
NIOSH	National Institute for Occupational Safety and Health

NLA	National Lakes Assessment
NMR	Nuclear magnetic resonance
NOAA	National Oceanic and Atmospheric Administration
NOAEL	No-observed-adverse-effect level
NRC	National Research Council
NRPS	Nonribosomal peptide synthetase
NTP	National Toxicology Program
OATp	Organic Acid Transporter polypeptides
OECD	Organization for Economic Cooperation and Development
OR	Odds ratio
PKS	Polyketide synthase
PND	Postnatal day
PMN	Polymorphonuclear leukocyte
POD	Point of departure
PP2A	Protein phosphatase 2A
PP1	Protein phosphatase 1
PSTs	Paralytic shellfish toxins
RBC	Red blood cell
RfD	Reference dose
ROS	Reactive oxygen species
RR	Relative risk
SDWA	Safe Drinking Water Act
SE	Standard error
SENCAR	SENsitivity to CARcinogenesis
SHE	Syrian hamster embryo
SOD	Superoxide dismutase
SRR	Standardized rate ratios
STX	Saxitoxin
TDI	Total Daily Intake
TEF	Toxicity equivalency factors
THP	Tamm-Horsfall protein
TWA	Time-weighted average
UF	Uncertainty factor
USGS	United States Geological Survey
UV	Ultraviolet
WHO	World Health Organization

1.0 EXECUTIVE SUMMARY

To be prepared after peer review

2.0 IDENTITY: CHEMICAL AND PHYSICAL PROPERTIES

Cyanobacteria or blue-green algae (Cyanophyceae) are a group of microorganisms comprising unicellular to multicellular prokaryotes that possess chlorophyll a and are capable of carrying out the light and dark phases of photosynthesis (Castenholz and Waterbury, 1989). Although most cyanobacteria are aerobic photoautotrophs, requiring only water, carbon dioxide, inorganic nutrients, and light for survival, some have heterotrophic properties and can survive long periods in complete darkness (Fay, 1965). Many species are capable of nitrogen fixation (diazotrophs) (Duy et al., 2000) leading to the production of inorganic nitrogen compounds that can be used to synthesize nitrogen-containing biomolecules such as nucleic acids and proteins. Cyanobacteria can form symbiotic associations with animals and plants, such as fungi, bryophytes, pteriodophytes, gymnosperms and angiosperms (Rai, 1990), providing interactive relationships that support their growth and reproduction.

Cyanobacteria are present in unicellular, colony and multicellular filamentous forms. Unicellular forms occur when the daughter cells separate after reproduction by binary fission. These cells can aggregate into irregular colonies, held together by a slimy matrix secreted during the growth of the colony (WHO, 1999). The filamentous form is the result of repeated cell divisions occurring in a single plane at right angles to the main axis (WHO, 1999). Reproduction is asexual.

Under the right conditions of temperature, light, pH, nutrient availability, etc., cyanobacteria can reproduce rapidly leading to the formation of a bloom. Humic substances, such as fulvic acids and humic acids, have been reported to stimulate algal growth (Kosakowska et al., 2007). Studies have found that presence of iron along with humic substances increases the formation of *Microcystis aeruginosa* blooms, especially in eutrophic[1] environments. Environmental factors contributing to the production of toxin in cyanobacteria are poorly understood (Duy et al, 2000).

In addition to chlorophyll a, cyanobacteria contain carotene, xanthonyll, blue *c* phycocyanin and red *c* phycoerythrin (Duy et al, 2000). They stain as gram negative indicating the presence of a complex cell wall. They produce a wide range of toxins as secondary metabolites also known as cyanotoxins. The cyanotoxins fall into three broad groupings: cyclic peptides, alkaloids and lipopolysaccharides (LPS). Table 2-1 provides an example of specific toxins within these groups and Table 2-2 provides chemical and physical properties of the three primary cyanotoxins (microcystin, anatoxin-a, cylindrospermopsin) discussed in this report.

Lipopolysaccharides (LPS) are the last major grouping of cyanotoxins and the least understood. They are irritants generally found in the outer cell wall of gram-negative bacteria including cyanobacteria. However, compared to gram-negative bacteria LPS, little is known about the chemical and structure activity of cyanobacterial LPS (Jaja-Chimedza et al., 2012). The LPS are usually comprised of a sugar moiety (generally a hexose), and a lipid moiety (normally a hydroxylated C14 to C18 fatty acid.) Cyanobacterial LPS endotoxins generally contain small amounts of phosphate (Duy et al., 2000). The diversity of LPS structures is largely related to phylogeny. Therefore, each cyanobacterial LPS genus has a distinct LPS composition.

Commented [IS1]: This classification scheme comprising cyclic peptides, alkaloids and LPS is arguably not particularly helpful, particularly wrt the secong group: alkaloids. The alkaloid cyanotoxins comprise structurally unrelated (beyond the obvious observation that they're alkaloids) and functionally diverse compounds. As for lipopolysaccharides, some have argued that there's not good evidence that cyanobacterial LPS should be considered toxins in their own right (see Stewart I, Schluter PJ, Shaw GR (2006) *Environ Health* 5:7). Many cyanobacteriologists classify these toxins by their primary functional properties, i.e. hepatotoxins, neurotoxins, dermal toxins. Of course, there are weaknesses with a functional classification, particulary wrt how cylindrospermopsin fits into it (some call it a "general cytotoxin" which again doesn't tell the reader much). Perhaps some mention of other classification approaches might be pertinent here?

Commented [IS2]: This citation not in bibliography

[1]Waters rich in mineral and organic nutrients that promote a proliferation of plant life, especially algae.

Recent studies have shown differences in the toxicity between cyanobacterial and heterotrophic LPS. Cyanobacterial LPS have been described as weakly toxic compared to heterotrophic bacteria LPS (Stewart et al., 2006). Studies have suggested that gram-negative bacteria LPS may be linked to a variety of health effects in humans including gastrointestinal and dermatological effects and allergic reactions. However, the health implications of cyanobacterial LPS are poorly understood and further studies are required to clarify how chemical composition and structure differences may relate to endotoxin activity.

Commented [IS3]: What studies? Reference/s needed here. The review of cyanobacterial LPS (not cited in the report under review) by Stewart et al (*Environ Health* 5:7, 2006) called into question those somewhat vague suggestions, repeated in the sentence "Studies have suggested…" and argued that there is not good evidence that heterotrophic bacterial LPSs are the causative agents, by natural exposure routes, of S&S in humans such as G-I, dematological and allergic reactions. And likewise there's not good evidence that cyanobacterial LPS are any more likely than bacterial LPSs alone to be fingered for these acute illnesses.

Table 2-1. Cyanotoxins and Cyanobacteria Genera Producing Toxins (adapted from Table 3-1, WHO, 1999 and Codd et al, 2005)

Cyanotoxin Group[1]	Cyanotoxin	Cyanobacteria Genera Producing toxin[2]
Cyclic Peptides	Microcystins	*Microcystis, Anabaena, Planktothrix (Oscillatoria), Nostoc, Hapalosiphon, Anabaenopsis, Aphanizomenon*
	Nodularin	*Nodularia*
Alkaloids	Anatoxin-a	*Anabaena, Planktothrix (Oscillatoria), Aphanizomenon, Cylindrospermopsis*
	Anatoxin-a (S)	*Anabaena*
	Aplysiatoxins	*Lyngbya, Schizothriz, Planktothrix (Oscillatoria)*
	Cylindrospermopsins	*Cylindrospermopsis, Aphanizomenon, Umezakia, Anabaena, Lyngbya, Rhaphidiopsis*
	Lyngbyatoxin-a	*Lyngbya*
	Saxitoxins	*Anabaena, , Aphanizomenon, Lyngbya, Cylindrospermopsis*
Lipopolysaccharides		All genera

[1] Many structural variants may be known for each toxin group
[2] Not all species of a particular genus produce the specific toxin

Commented [IS4]: This table could be updated? If it is not intended to be an exhaustive listing of toxin-producing genera, it should be stated as such, maybe in the text that introduces the Table? Otherwise there's another nodularin-producing genus (the name of which escapes me right now), *Phormidium* (antx-A), *Planktothrix* as a saxitoxin producer, and others.

Table 2-2. Chemical and Physical Properties of Microcystin-LR, Anatoxin-a and Cylindrospermopsin

Property	Microcystin-LR	Anatoxin-a	Cylindrospermopsin
Chemical Abstracts Registry (CAS) #	101043-37-2	64285-06-9	143545-90-8
Chemical Formula	$C_{49}H_{74}N_{10}O_{12}$	$C_{10}H_{15}NO$	$C_{15}H_{21}N_5O_7S$
Molecular Weight	995.17 g/mole	165.23 g/mole	415.43 g/mole
Color/Physical State	solid	lyophilized solid	white powder
Boiling Point	N/A	291°C at 760 mmHg	N/A
Melting Point	N/A	N/A	N/A
Density	1.29 g/cm^3	1.037 g/cm^3	2.03g/cm^3
Vapor Pressure at 25°C	N/A	0.002 mmHg	N/A
Henry's Law Constant	N/A	N/A	N/A
K_{ow}	N/A	N/A	N/A
K_{oc}	N/A	N/A	N/A
Solubility in Water	Highly	t≤50 mM	Highly
Other Solvents	Ethanol and methanol	N/A	Dimethylsulfoxide (DMSO) and methanol

Sources: Chemical Book, 2012; TOXLINE, 2012

2.1 Microcystins

The cyclic peptides include six variants of nodularins and more than 80 variants of microcystins. Figure 2-1 provides the structure of the microcystins where X and Y represent variable amino acids as presented in Table 2-3. The amino acids are joined end-to-end and then head to tail to form cyclic compounds that are comparatively large, (molecular weights ranging from ~ 800 to 1,100 g/mole).

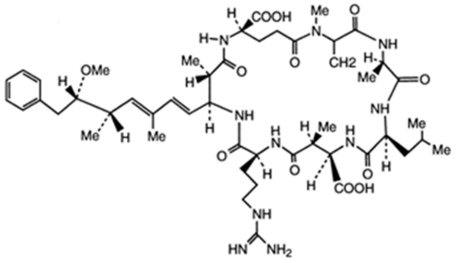

Figure 2-1. Structure of Microcystin-LR (Kaebernick and Neilan, 2001)

Microcystin congeners vary based on their amino acid composition and through methylation or demethylation at selected sites within the cyclicpeptide (Duy et al, 2000). The variations in composition and methylation account for the large number of toxin variants. The

microcystins are named based on their two variable amino acids. For example, microcystin-LR, the most common congener, contains leucine (L) and arginine (R) (Carmichael, 1992). The letters used to identify the variable amino acids are the standard single letter abbreviations for the amino acids found in proteins. The variable amino acids are usually the L- amino acids as found in proteins. There has been at least one microcystin where the leucine was D-leucine.

In this report the microcystins will be abbreviated with the initials MC for microcystins followed by the abbreviations for the variable amino acids. For example, MC-LR for the microcystin with leucine in the X position of Figure 2-1 and arginine in the Y position. Most research has concentrated on microcystin-LR (MC-LR) with lesser amounts of data available for the other amino acid combinations.

Table 2-3. Abbreviations for Microcystins (Yuan et al., 1999)

	Amino Acid in X	Amino Acid in Y
Microcystin-LR	Leucine	Arginine
Microcystin-RR	Arginine	Arginine
Microcystin-YR	Tyrosine	Arginine
Microcystin-LA	Leucine	Alanine
Microcystin-LY	Leucine	Tyrosine
Microcystin-LF	Leucine	Phenylalanine
Microcystin-LW	Leucine	Tryptophan

Structurally, the microcystins are monocyclic heptapeptides that contain seven amino acids: two variable L-amino acids, three common D-amino acids or their derivatives, and two novel D-amino acids (Adda and Mdha). Adda (3-amino-9-methoxy-2,6,8,-trimethyl-10-phenyldeca-4,6-dienoic acid) is characteristic of all toxic microcystin structural variants and is essential for their biological activity (Lakshmana et al., 2002; Funari and Testai, 2008). Mdha (methyldehydroalanine) is the second unique component of the microcystins. It plays an important role in the ability of the microcystins to inhibit protein phosphatases. Figure 2-2 illustrates the structures of the two unique amino acid microcystin components.

Microcystins are water soluble. In aquatic environments the cyclic peptides tend to remain contained within the cyanobacterial cell and are released in substantial amounts only upon cell lysis. The microcystins are most frequently found in cyanobacterial blooms in fresh and brackish waters where they are chemically stable (WHO, 1999).

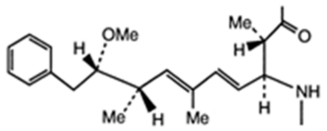

Figure 2-2. Structure of the amino acid Adda (Kaebernick and Neilan, 2001)

2.2 Anatoxin-a

The alkaloid cyanotoxins are a broad group of heterocyclic nitrogenous compounds of usually low-to-moderate molecular weight (< 1000 g/mole; WHO, 1999). The available chemical structures for anatoxin-a and homoanatoxin-a are presented in Figure 2-3. The alkaloid toxins are produced by plants and some bacteria. They include the neurotoxic non-sulfated toxins (anatoxin-a, homoanatoxin-a, anatoxin-a(S), and saxitoxin [STX]) produced by freshwater bacteria as well as the neurotoxic sulfated derivatives of saxitoxin (sulfated Paralytic Shellfish Poisons [PSPs], C-toxins, and gonyautoxins [GTX]); the hepatotoxic sulfated alkaloid cylindrospermopsin; and the dermatotoxic alkaloids lyngbyatoxins, and aplysiatoxins produced by benthic marine cyanobacteria (WHO, 1999).

Anatoxin-a is a tropane-related[2] bicyclic alkaloid (Duy et al, 2000). The presence of a methyl group on carbon atom 11 is the only difference between anatoxin-a and homoanatoxin-a (Figure 2-3). Both molecules share almost identical toxicological properties (Funari, 2008). Anatoxin-a has a molecular formula of $C_{10}H_{15}NO$ and a molecular weight of 165.26 g/mole (Lewis, 2000). Its chemicals name is 2-acetyl-9-azbicyclo[4:2:1]non-2-ene.

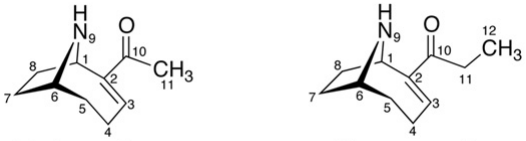

Figure 2-3. Structures of Anatoxin-a and Homoanatoxin-a (Mann et al., 2012)

2.3 Cylindrospermopsin

Cylindrospermopsin (Figure 2-4) is a tricyclic alkaloid with the following molecular formula ($C_{15}H_{21}N_5O_7S$; Funari and Testai, 2008). Cylindrospermopsin is water soluble as well as stable in extreme temperatures and pH (Moore et al., 1998, Chiswell et al., 1999). It is zwitterionic (i.e., a dipolar ion with localized positive and negative charges) (Ohtani et al., 1992) and is believed to be derived from a polyketide that uses an amino acid starter unit such as glycocyamine or 4-guanidino-3-oxybutyric acid (Duy et al., 2000).

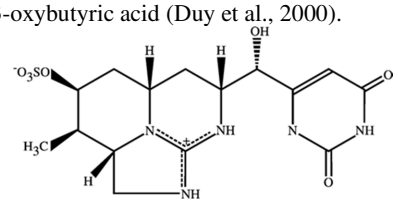

Figure 2-4. Structure of Cylindrospermopsin (Wiegand and Pflugmacher, 2005)

[2] A nitrogenous bicyclic heterocycle.

Commented [IS5]:

Commented [IS6]: intermediate?

Commented [IS7]: Structure shown is 7-epi cylindrospermopsin. For definitive stereochemistry of CYN, see Looper, Runnegar & Williams (2006) *Tetrahedron* 62:4549-62; also Heintzelman GR *et al* (2002) *J Am Chem Soc* 124:3939-45

3.0 TOXIN SYNTHESIS AND ENVIRONMENTAL FATE

The biosynthetic pathways of cyanotoxins are in the early stages of investigation. No complete biochemical pathways are known, but the available data are summarized in Section 3.1. The environmental fate of cyanobacteria in surface water and water treatment is summarized in Section 3.2.

Commented [IS8]: This isn't my field, so I may stand corrected here, but perhaps this particular aspect (cyanotoxix biosynthesis) might benefit from a more recent review. I seem to recall several recent papers on this topic, with more work done on cylindrospermopsin biosynthesis…

3.1 Toxin Synthesis

Toxin production varies between blooms and within an individual bloom over time (Duy et al., 2000). Cyanotoxins can be produced by more than one cyanobacterial species and species may produce more than one toxin at a time, resulting in blooms with different cyanotoxin combinations (Funari and Testai, 2008). The toxicity of a given bloom is determined by the mixture of species involved and their strain composition of toxic and nontoxic genotypes (Chorus, 2001). Generally, toxins in cyanobacteria are retained within the cell unless conditions favor cell wall lysis (ILS, 2000).

Cyanotoxins synthesis is poorly understood but evidence suggests that the production and accumulation of toxin(s) correlates with the cyanobacterial growth rate, with the highest amount being produced during the late logarithmic phase (Funari and Testai, 2008). For example, Long (2000) described a positive linear relationship between the content of microcystin in cells and their specific growth rate (Long et al., 2000). Sukenic (1998) found that the concentration of cylindrospermopsin within *A. ovalisporum* from Lake Kinneret increased to a plateau during the growth phase and then decreased during the stationary phase. The authors attributed this decrease to cell degradation and the release of the water-soluble toxin into the medium. Saker and Neilan (2001) observed the highest concentration (on a dry-weight basis) of cylindrospermopsin in cultures of *Cylindrospermopsis raciborskii* in the absence of a fixed nitrogen source (Saker and Neilan, 2001).

3.1.1 Microcystins

Evidence suggests that the environmental conditions in which a bloom occurs may alter the levels of toxin produced. Several culture experiments have suggested that the biosynthesis of microcystin is regulated by environmental and nutritional factors including light intensity, temperature, and nutrients such as nitrogen, phosphorus, and iron (Neilan et al., 2007). However, the physiological function of iron is still unclear. Studies on the effect of different light intensities on microcystin production have come to contradictory conclusions (Neilan et al., 2007).

Although there is little information on the genetic regulation of microcystin production, Dittman et al. (1997) showed that peptide synthetase genes are responsible for microcystin production. Studies conducted by Kaebernick et al. (2000) on *Microcystis aeruginosa* suggest that microcystin is produced nonribosomally through large multifunctional enzyme complexes consisting of both nonribosomal peptide synthetase (NRPSs) and polyketide synthase (PKS) modules coded by the *mcy*S (microcystin) gene cluster. According to Gewolb (2002), most

NRPSs are made up of a series of four to 10 modules, each of which is responsible for specific steps for activation, modification, and condensation during the addition of one specific amino acid or other compound to the growing linear peptide chain that is then cyclized to produce microcystin. The sequence of modules in an enzyme determines the type of microcystin produced (Gewolb, 2002).

The difference in toxicity of microcystin variants depends on the amino acid composition (Falconer, 2005). The most toxic microcystins are those with the more hydrophobic L-amino acids (-LA, -LR, -YR and -YM) and the least toxic are those with hydrophilic amino acids, such as microcystin-RR. The Adda group is also important since its removal or saturation of its double bonds greatly reduces toxicity.

3.1.2 Anatoxin-a

Little is known of the biosynthesis of anatoxin-a. Enzymatic studies and analysis of the incorporation of labeled precursors have found that the carbon skeleton of anatoxin-a is derived from acetate and glutamate. Studies showed that C-1 of glutamic acid is retained during the formation of anatoxin-a and is not lost by decarboxylation. Recent studies have proposed that the biosynthesis of anatoxin-a genes would encode a mixed PKS/NRPS system for activating an amino acid of the glutamic acid family followed by the incorporation of three acetates in a polyketide manner (Neilan et al, 2007).

3.1.3 Cylindrospermopsin

Moore et al. (1993) suggested that a mixed non-ribosomal peptide and polyketide may be the precursors for cylindrospermopsin. Its biosynthesis starts with the production of guanidinoacetate from glycine and arginine, a natural guanidino donor, followed by successive condensations of five intact acetates, and subsequently by methylation, ketoreduction, sulfation, and cyclizations. Enzymes encoded by two genes (*cyrA*- and *cyr0*) have been detected in cylindrospermopsin-producing strains of *C. raciborskii* and are believed to initiate toxin biosynthesis (Schembri et al., 2001; O'Neil et al., 2012).

Little is known about the effect of nitrogen on cylindrospermopsin production. However, some studies have suggested that increased intracellular cylindrospermopsin content in the absence of fixed nitrogen was due to hyp gene homologs in the *C. raciborskii* genome associated with the maturation of hydrogenases (O'Neil et al., 2012). Phosphorus appears to play an important role in cylindrospermopsin production by *C. raciborskii* due to the presence of genes to utilize inorganic and organic P, including those for high affinity phosphate binding proteins (*pstS* and *sphX*), phosphanate transport proteins (*phnC,D,E*), and enzymes for metabolism (*phnG-M,X,W* and *phoA*).

3.2 Environmental Fate of Cyanotoxins

3.2.1 Cyanotoxin Production in Surface Water

Cyanotoxin production is strongly influenced by the environmental conditions that promote growth of particular cyanobacterial species and strains. Micronutrient concentrations, light intensity, temperature, competing bacteria and phytoplankton, pH, turbulence, and salinity are all factors that affect growth and change in cyanobacteria population dynamics. Although environmental conditions affect the formation of blooms, the numbers of cyanobacteria and toxin concentrations produced are not always closely related. Cyanotoxin concentrations depend on the dominance and diversity of strains within the bloom along with environmental and ecosystem influences on bloom dynamics (Hitzfeld et al., 2000; Chorus, 2001).

Commented [IS9]: Also turbidity?

Nutrients

Nutrient concentrations are considered to be the key driving environmental factor influencing the proportion of cyanobacteria in the phytoplankton community, the cyanobacterial biovolume, and the impact that cyanobacteria may have on ecosystem functioning and water quality. Nutrient concentrations and nitrogen (N) and phosphorus (P) bioavailability have varying influences on cyanobacteria species dominance and toxin production. Studies have found that cyanobacteria production and toxin concentrations are dependent on nutrient levels (Wang et al., 2002), but that different cyanobacteria species use organic and inorganic nutrient pools differently. N loading can enhance the growth and toxin levels of *Microcystis sp.* blooms and microcystin synthetase gene expression (Gobler, et al., 2007; O'Neil et al., 2012). Gobler et al. (2007) suggest that dominance of *Microcystis sp*. blooms during summer is linked to N loading, which stimulates growth and toxin synthesis. This may cause the inhibition of grazing by mesozooplankton and further accumulation of algal cells.

Commented [IS10]: cyanobacterial ? (insofar as cyanobacteria are not algae…)

Loading of N and/or P in water bodies provides large amounts of essential nutrients needed for the development of algal blooms and cyanotoxin production (Paerl et al., 2011). Optimal concentrations of total and dissolved phosphorous (Wang et al., 2002) and soluble phosphates and nitrates (ILS, 2000; Paerl and Scott, 2010; Wang et al., 2010; O'Neil et al., 2012) may result in the increased production of microcystins. Some studies have observed a decrease in toxicity of *Microcystis sp.* after removal of N or inorganic carbon, but no changes were observed when P was removed from a cyanobacteria culture media (Codd and Poon, 1988). Similarly, Sivonen (1990) found a relationship between high toxicity and high N concentration, but no effect at higher concentrations of P.

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Smith (1983) first described a strong relationship between the relative amounts of N and P in surface waters and cyanobacterial blooms. Smith proposed that diazotrophic cyanobacteria should be superior competitors under conditions of N-limitation because of their unique capacity for N-fixation. The hypothesis that low N:P ratios favor cyanobacteria has been intensely debated (Lampert, 1999) and challenged for its poor performance predicting cyanobacterial dominance (Downing et al., 2001). However, the dominance of N-fixing cyanobacteria at low N:P ratios has been demonstrated in mesocosm- and ecosystem-scale experiments in prairie and boreal lakes (Schindler et al., 2008, and references therein). Nonetheless, there is debate whether

these experimental results can be generalized to hypereutrophic lakes with a long history of anthropogenic nutrient loading (e.g., Paerl et al., 2011).

Recently, Orihel et al. (2012) investigated the relationship between the mass ratio of N to P and microcystin concentrations in 246 water bodies across Canada. Given that production of microcystins is exclusive to certain members of the phylum Cyanophyta (Cyanobacteria), the presence of microcystins in lakes should theoretically be higher under low N:P ratios if cyanobacteria dominate under conditions of relative N deficiency. It is known that some cyanobacteria that produce microcystins are capable of N-fixation (e.g., *Anabaena*), but many microcystin producers are non-diazotrophic (e.g., *Microcystis*). The dominance of cyanobacteria does not necessarily predicate the occurrence of microcystins because not all cyanobacterial species are capable of synthesizing microcystins and not all strains of known toxin-producing species are toxic. As such, this hypothesis does not imply that a simple, linear, negative relationship exists between N:P ratios and microcystin concentrations, but rather that microcystin concentrations can be potentially elevated at low N:P ratios. The results from Orihel et al. supported the hypothesis that microcystin concentrations increase with lake trophic status (Kotak and Zurawell, 2007). Microcystin concentrations in the Canadian lakes sampled were positively correlated with concentrations of N (Spearman rank correlation, $r = 0.39$, $p < 0.01$, $n = 937$) and P (Spearman rank correlation, $r = 0.36$, $p < 0.01$, $n = 937$). However, the authors state that the strength of these correlations imply that variables other than nutrient concentrations most certainly played a role in determining microcystin concentrations (Orihel et al., 2012).

In addition, Orihel et al. (2012) calculated minimum thresholds for N and P (independently) as the nutrient concentration above which 95% of values at a specific level of microcystin occurred (e.g., the WHO drinking water guideline of 1 µg/L). These correlation analyses and minimum threshold determinations demonstrated that microcystins were primarily a concern in eutrophic and hypereutrophic systems. The meta-analysis showed that microcystin concentrations in Canadian fresh waters were high only at low N:P ratios and were consistently low at high N:P ratios. The authors propose the use of N:P ratios for estimating the "risk" of elevated microcystin concentrations but caution that N:P ratios are not suitable for predicting absolute concentrations of microcystins. Likewise, a "cause-and-effect" argument is not supported by the measured data in this study, nor are the causal mechanisms identified to explain why high microcystin concentrations coincide with low N:P ratios (Orihel et al., 2012). Subsequent experiments should manipulate N:P ratios at scales relevant to ecosystem management as the outcome may be germane to the ongoing debate regarding the need for a "dual-nutrient management strategy" as discussed in Paerl et al. (2011).

Commented [IS12]: Caution advised in the use of the term "meta-analysis" Meta-analysis came from the field of epidemiology, and involves firstly doing a systematic review, which informs the meta-analysis, this being a formal statistical evaluation of studies that meet the selection criteria. I'm one of those scientists that resists the tendency to water-down the principles of meta-analysis, as exemplified by a group of cyanobacteriologists who published something called a "qualitative meta-analysis…"

Light Intensity

Sunlight availability and turbidity have a strong influence on the cyanobacteria species that predominate and the depth at which they occur (Falconer, 2006; Carey et al., 2012). For example, *Cylindrospermopsis* forms dense layers of filaments at depths toward the lower bound of the euphotic zone in deeper rivers, lakes, and reservoirs. On the other hand, *Microcystis aeruginosa* occurs mostly at the surface with higher light intensities and in shallow lakes. The relationship of light intensity to toxin production in blooms is somewhat unclear and continues to be investigated (Duy et al., 2000). Some scientists have found evidence that toxin production

increases with high light intensity (Watanabe and Osihi, 1985), while others have found little variation in toxicity at different levels of light intensity (Codd and Poon, 1988).

Recently, Kosten et al (2011) discussed results from sampling of 143 lakes along a latitudinal transect ranging from subarctic Europe to southern South America (between 5-55°S and 38-68°N). One finding from these analyses was that the percentage of the total phytoplankton biovolume attributable to cyanobacteria is greater in lakes with high rates of light absorption. This observation points to a positive feedback because restriction of light availability is often a consequence of high phytoplankton biovolume, which in turn may be driven by nutrient loading. Kosten et al note that although cause and effect cannot be established from these field data other controlled experiments and field data also suggest that light availability might affect the competitive balance among a large group of shade-tolerant species of cyanobacteria, mainly *Oscillatoriales*, and other phytoplankton species (Smith, 1986; Scheffer et al., 1997). Overall, results from the Kosten et al study suggest that higher temperatures interact with nutrient loading and underwater light conditions in determining the proportion of cyanobacteria in the phytoplankton community of the shallow lakes sampled.

Temperature

The increasing body of laboratory and field data (Weyhenmeyer, 2001; Huisman et al., 2005; Reynolds, 2006; De Senerpont Domis et al., 2007; Jeppesen et al., 2009; Wagner & Adrian, 2009; Kosten et al., 2012; Carey et al., 2012) suggest that warming may promote cyanobacterial dominance. Cyanobacteria may benefit more from warming than other phytoplankton groups due to their higher optimum growth temperatures. The optimum temperatures for microcystin production range from 20 to 25°C (WHO, 2003). Likewise, the increase in water column stability that is associated with higher temperatures supports cyanobacteria dominance in the phytoplankton community (Wagner and Adrian, 2009; Carey, 2012). The key result from the Kosten et al. (2011) (analyses of 143 lakes along a latitudinal transect ranging from subarctic Europe to southern South America) is that the percentage of the total phytoplankton biovolume attributable to cyanobacteria increased steeply with temperature in the shallow lakes sampled during the summer.

Indirectly, warming may also increase nutrient concentrations by enhancing mineralization (Gudasz et al., 2010; Kosten et al., 2010) and by temperature or anoxia-mediated sediment phosphorus release (Jensen and Andersen, 1992; Søndergaard et al., 2003). This implies that temperature may indirectly increase cyanobacteria biomass through its effect on nutrient concentrations. Others have suggested warmer conditions may raise total phytoplankton biomass through an alteration of top-down regulation by grazers (Jeppesen et al., 2009, 2010; Teixeira-de Mello et al., 2009). The relationship between temperature and cyanobacterial dominance may be explained not only from temperature effects on the competitive advantage for cyanobacteria but also from a set of temperature-induced mechanisms that alter underwater light levels favorably for cyanobacteria (Kosten et al., 2011; Carey et al., 2012).

Other Environmental Factors

Cyanobacterial blooms have been shown to persist at pH levels between six and nine (WHO, 2003). Kosten et al. (2011) also found on their lake studies in Europe to southern South America that pH was the next most important variable after temperature in determining

cyanobacteria abundance. The percentage of cyanobacteria in these shallow lakes was well correlated with pH. Cyanobacteria are efficient users of molecular carbon dioxide (Shapiro, 1984; Caraco and Miller, 1998) which is less available in the water column with increasing pH. Although this could explain the positive relation found between pH and the proportion of cyanobacteria, the high proportion of cyanobacteria at high pH may also arise from an indirect nutrient effect. The pH increases as photosynthesis intensifies due to inorganic carbon uptake from the water. The high pH therefore often reflects high photosynthetic rates, which are linked with high nutrient concentrations. Trace metals like iron and zinc have also been known to affect toxin production (Duy et al., 2000). High iron concentrations (more than 100 µM) increase cell density and chlorophyll content in *Microcystin aeruginosa* (Kosakowska et al., 2007).

The concentration of cyanotoxins in water when a bloom collapses from aging or by treatment with algicides depends on dilution of the toxin in the impacted water, water column mixing, the degree of adsorption to sediment, and biodegradation (Funari and Testai, 2008). In addition, the vertical phytoplankton biomass structure and cyanotoxin production can be influenced by seasonal changes as well as weather conditions (wind, rainfall, runoff). Most blooms occur in late summer and early fall, thus changing the vertical stratification of the phytoplankton community. At times, the bottom layer can have more biomass and display different population dynamics than the upper water column. Conversely, seasonal influences with increases in temperature and changes in wind patterns may favorably influence the upper water column cyanobacterial community to become dominant. This vertical variability is common and attributed to four causes, each of which may occur at different times: (a) sinking of dead/dying cells; (b) density stratification of the water column, which affects all aspects of the cyanobacteria growth, especially nutrient concentrations and light; (c) nutrient supply from the organic-rich bottom sediments even when the water body is not density-stratified, encouraging growth at or near the bed; and (d) species-specific factors, such as the tendency to form surface scums in the case of *M. aeruginosa* or the presence of resting spores in the sediment in the case of *N. spumigena* (Drake et al., 2010).

3.2.2 Fate of Cyanotoxins in Surface Water

3.2.2.1 Environmental Fate of Microcystins

Hydrolysis Microcystins are extremely stable and resistant to chemical hydrolysis or oxidation at near neutral pH. Elevated or low pH or temperatures above 30°C may cause slow hydrolysis. They have been reported to remain potent toxins even after boiling (Lakshmana et al., 2002). In natural waters kept in the dark, microcystins may persist for times ranging between 21 days and 2-3 months in solution and up to 6 months in dry scum (Rapala et al., 2006; Funari and Testai, 2008).

Photolysis In the presence of full sunlight, microcystins undergo slow photochemical breakdown, but this varies by microcystin strain (Chorus, 2001). The presence of water-soluble cell pigments, in particular phycobiliproteins, enhances this breakdown. Breakdown can occur in as little as two weeks or longer than six weeks depending on the concentration of pigment (Tsuji

Commented [IS13]: Is "strain" the correct term here, rather than variant, congener, perhaps analogue?

et al., 1993). Humic substances can also act as photosensitizers and increase the rate of microcystin breakdown under sunlight. In deeper or muddier water, the breakdown rate is slower.

Metabolism Although microcystins may be stable to abiotic breakdown, they are susceptible to degradation by aquatic bacteria found naturally in rivers and reservoirs (Jones et al., 1994). Bacteria isolates of *Arthrobacter*, *Brevibacterium*, *Rhodococcus*, *Paucibacter*, and various strains of the genus *Sphingomonas (Pseudomonas)* have been reported to be capable of degrading microcystin-LR (de la Cruz et al., 2011; Han et al., 2012). The degradative bacteria have also been found in sewage effluent (Lam et al., 1995), lake water (Jones et al., 1994; Cousins et al., 1996; Lahti et al., 1997a), and lake sediment (Rapala et al., 1994; Lahti et al., 1997b).

Transport Microcystins may adsorb onto naturally suspended solids and dried crusts of cyanobacteria. They can precipitate out of the water column and reside in sediments for months (Han et al., 2012: Falconer, 1998). A study by USGS and the University of Central Florida determined that the saturated groundwater flow transport of microcystin and cylindrospermopsin in sandy aquifers resulted in little adsorption (O'Reilly et al., 2011). The authors suggested that the removal of microcystin was due to biodegradation.

3.2.2.2 Environmental Fate of Anatoxin-a

Hydrolysis Alkaline conditions accelerate anatoxin-a breakdown (Stevens and Krieger, 1991a).

Photolysis Anatoxin-a is relatively stable in the dark. However, anatoxin-a differs from microcystin in that it undergoes rapid photochemical degradation in sunlight even in the absence of cell pigments (WHO, 1999). Sunlight can cause isomerization of the double bonds and hydroxylation in the presence of pigments. Anatoxin-a showed a half-life of about 14 days in normal light conditions with basic pH and low initial concentrations, but showed a shorter half-life (1-2 hours) in the presence of high light intensity (Funari and Testai, 2008).

Metabolism Anatoxin-a may be readily degraded by bacteria associated with cyanobacterial filaments. A five-day half-life was measured in samples of lake sediment and natural bacteria in the laboratory (Smith and Sutton, 1993)

Transport In sediments, anatoxin-a is absorbed by a cation-exchange mechanism when there is higher clay and organic carbon content (Klitzke et al., 2011).

Commented [IS14]: adsorbed?

3.2.2.3 Environmental Fate of Cylindrospermopsin

Hydrolysis Cylindrospermopsin is relatively stable to heat (4 to 50°C), pH, and light (Moore et al, 1998). Cylindrospermopsin remains a potent toxin even after boiling for 15

minutes. Cylindrospermopsin is stable at temperatures ranging up to 50°C for 5 weeks in the dark (ILS, 2000).

Photolysis Like microcystin, pure cylindrospermopsin is relatively stable in the sunlight, but in the presence of cell pigments, photochemical degradation can occur more rapidly. Photolysis can break down more than 90% of cylindrospermopsin within 2-3 days dependent upon the presence of cell pigments (Chiswell et al, 1999).

Metabolism A half-life of 11 to 15 days has been reported for cylindrospermopsin in surface waters (Funari and Testai, 2008). Blooms of *C. raciborskii* have been reported to increase at a pH of 8.4 to 9.0 and cylindrospermopsin toxin can remain stable at pH of 4, 7, and 10 for a period of up to 8 weeks (ILS, 2000). No laboratory data were found on the biodegradation of cylindrospermopsin by bacteria, however toxins released into lakes may be decomposed by bacteria (Falconer, 1998).

Commented [IS15]: Might be worth an updated search? I know folk have been working on biodegradation of CYN, but I'm not sure if they've published or not…

Transport No information on transport of cylindrospermopsin was identified.

3.2.3 Fate of Cyanotoxins during Water Treatment

Algicides are usually applied to lakes and reservoirs to suppress cyanobacterial blooms. In most cases, the resulting cell lysis releases toxins to impacted source waters (Han et al., 2012; Falconer, 1998). Compounds (e.g., alum, copper compounds, straw) to control blooms in drinking water sources can only be used safely if the bacterial concentration is still relatively low and it is early in the life cycle of the cyanobacterial bloom. In such cases, it is recommended that another source of water be made available until treatment residues are no longer of a concern in the municipal water source (Westrick, 2010).

One of the more effective methods of algae control in reservoirs is the use of aeration and destratification (Falconer, 1998). In lakes and source water reservoirs, long-distance circulation equipment has been deployed to treat the epilimnion (above the thermocline) with a goal of controlling cyanobacteria blooms. Methods like solar powered circulation pumps have been proven to be effective in suppressing harmful algal blooms (Hudnell et al., 2010) in smaller water bodies from one acre to several tens of acres. Circulation treatments can be to the entire lake to just a portion of the lake (partial lake treatment) depending on the need, size and depth of lake to be treated, and the intended use of the waterbody.

For surface waters that are sources of drinking water, physical engineering methods like positioning water intakes away from areas where blooms are known to occur (e.g., sheltered bays) or accumulate (usually downwind of prevailing winds), and barriers to restrict scum movement may be recommended to reduce the amount of cyanobacteria taken into the treatment plant with the source water. Positioning the depth of source water intake at a location where the cyanobacterial concentrations are lower (e.g., subsurface intakes) also reduces the possibility of cyanobacterial contamination (Westrick, 2010).

Effective treatment of cyanotoxins in drinking water includes evaluation and selection of appropriate treatment methods. The chemical preparation should use an adequate concentration of the chemical treatment and should be conducted at the appropriate pH. Some oxidants may be used before coagulation and clarification, but care must be taken not to cause cell lysis. The water-treatment method used needs to be tailored to the type(s) of cyanobacteria present and the environmental conditions of the raw-water storage facilities, as well as the physical and environmental conditions of the water-conveyance system and the water treatment facility. Drinking water treatment facilities that use microstrainers or fine screens to remove debris from the water intake are useful in removing larger algae, cyanobacterial cells, and aggregated cells (Mouchet and Bonnelye, 1998). Riverbank filtration has been demonstrated to be effective for removal of cyanotoxins except during massive algal blooms (Schmidt et al., 2003). Oxidants are often added at the intake to reduce taste and odor problems and to discourage biological growth (zebra mussels, biofilm, and algae) on the intake pipe. However, pretreatment oxidation is not recommended because it may rupture cyanobacteria cells releasing the cyanotoxin into the water column and cause the formation of chlorinated disinfection by-products (Westrick, 2010).

Conventional water treatment (flocculation, coagulation, sedimentation and filtration) is effective in removing algal cells and intracellular cyanotoxins (Chow et al., 1999; Jurczak et al., 2005; Rapala et al., 2006; Carrière et al., 2010). A study was done in 2005 to evaluate the prevalence of microcystin in drinking water supplies and distribution systems (Westrick et al., 2006). The study found that although four of the five conventional treatment plants experienced algal blooms in their source water, all of them effectively removed the algae and all distribution water samples showed microcystin levels below the detection limit of 0.05 µg/L. Removal of microcystins in water by coagulation and clarification can be little to moderately effective (42%) depending on initial toxin and co-occcuring organic compounds, especially during harmful bloom events (Lambert et al., 1996; Rapala et al., 2006; Jia et al., 2003; Schmidt et al., 2002; Hoeger et al., 2004; de la Cruz et al., 2011). Dissolved air flotation (DAF) is an effective treatment process for *Microcystis* and intracellular cyanotoxins removal (Westrick, 2010). Microfiltration and ultrafiltration are effective in removing intracellular toxins (Li et al., 2009; Dixon et al., 2011). Nanofiltration membranes have proven to be effective to remove 90% of microcystin-LR and dissolved cyanotoxins (Coral et al., 2011; Dixon et al., 2011).

Conventional water treatment is usually not effective in removing extracellular cyanotoxins (soluble toxins). Neither aeration nor air stripping are effective treatments for removing soluble toxins or cyanobacterial cells. Advanced treatment processes, such as powdered and granular activated carbon adsorption, must be implemented to remove extracellular toxins as well as intact cells (Westrick, 2010). Powdered activated carbon (PAC) has proven to be effective for removal of microcystin, anatoxin-a and cylindrospermopsin (Dixon et al., 2011). Granular activated carbon (GAC) is effective for microcystin based on the empty bed contact time, the carbon's age, and possible biodegradation of the toxin (Mohamed et al., 1999; Ho, 2004). GAC is less effective for anatoxin-a and cylindrospermopsin. The problem with the use of activated carbon is the quality and the doses of the carbon. Wood-based carbons have been identified to be more effective than coconut or peat-based carbons to remove microcystins, and high doses (30-100 mg/L) and contact times of 30 to 120 minutes are recommended (Carmichael, 1997b). The use of potassium permanganate ($KMnO_4$) has proven

to be effective for oxidizing microcystins and anatoxins, however, performance is highly dependent on potassium permanganate concentrations (Schmidt., 2002; Rositano et al., 1998).

Different cyanotoxins react differently to oxidants dependent on the individual characteristics of the source water such as Total Organic Carbon (TOC), temperature, and pH (Westrick, 2010; Sharma et al., 2012). While chlorination is an effective treatment for destroying microcystin and cylindrospermopsin, effectiveness is dependent on the pH (Merel et al., 2010). In bench-scale studies, chlorine proved to be effective (up to 100% removal) for microcystin M-LR where the effectiveness is dependent on chlorine dose, contact time, pH, temperature and water quality characteristics (Rapala et al., 2006; Nicholson et al., 1994; Rositano et al., 1998; Xagoraraki et al., 2006; Jia et al., 2003; Lambert et al., 1996; Tsuji et al., 1997). Data from full-scale water treatment plants indicate a much lower removal. Anatoxin-a is not degraded by chlorination. Other chlorine disinfectants such as chloramines and chlorine dioxide that are frequently used to minimize the formation of regulated disinfection by-products, have little impact on microcystin, cylindrospermopsin, and anatoxin-a (Westrick, 2010, Nicholson et al., 1994; Zamyadi et al., 2012). A dose of 20 mg/L of chloramine with a contact time of five days only to reduce the toxin concentration by 17% at room temperature (Nicholson et al., 1994). Therefore, water treatment utilities that use disinfectants other than chlorine in order to reduce the formation of disinfection by-products may not have an effective oxidant treatment for cyanotoxin inactivation.

Commented [IS16]: Sentence structure needs revising…

Other disinfection techniques like ozone and ultraviolet (UV) light have been shown to be effective in inactivating cyanotoxins (Kaya and Sano, 2005; Qiao et al., 2005; Pelaez et al., 2012). Ozone is a good oxidant of microcystin (Rositano et al., 2001; Brooke et al., 2006; Rapala et al., 2006), its efficacy is pH-dependent for anatoxin-a and cylindrospermopsin. These treatments are also only effective with sufficient contact time (Westrick, 2010). However, the possible formation of byproducts when an insufficient ozone dose is used are unknown (Funari and Testai, 2008). Ultraviolet (UV) is an effective treatment in destroying microcystin, anatoxin-a, and cylindrospermopsin cells (Westrick, 2010). However, it requires high dosages, making it a non-viable treatment for cyanotoxins. Because of variable and often high loads of dissolved organic carbon (DOC) during cyanobacterial blooms, frequent monitoring of treatment performance is required.

4.0 OCCURRENCE AND EXPOSURE IN WATER

The presence of detectable concentrations of cyanotoxins in the environment is closely associated with blooms (mass occurrences) of cyanobacteria. Cyanobacteria flourish in various natural environments including salty, brackish or fresh water, in cold and hot springs, and in environments where no other microalgae can exist, including infertile substrates, such as desert sand, on volcanic ash and rocks (Jaag, 1945; Dor and Danin, 1996). Cyanobacteria also form symbiotic associations with animals and plants, and the cyanotoxins may bioaccumulate in common aquatic vertebrates and invertebrates.

Currently, there is no national database recording freshwater harmful algal bloom (HAB) events. Instead states and local governments document HABs occurrence in various ways depending on the monitoring means and the availability of laboratories in the state capable of conducting algal toxin analyses.

4.1 Occurrence in Ambient Water

Cyanobacteria are predominantly found in eutrophic water bodies and are common in freshwater and marine environments (ILS, 2000). Most marine cyanobacteria grow along the shore as benthic vegetation between the low- and high-tide marks. The marine planktonic forms of cyanobacteria have a global distribution. Most species of cyanobacteria are highly adaptable and inhabit freshwater and highly saline environments such as salt marches. They are also found in hot springs (Castenholz, 1973; Mohamed, 2008), mountain streams (Kann, 1988), Arctic and Antarctic lakes (Skulberg, 1996) and snow and ice (Kol, 1968; Laamanen, 1996).

Commented [IS17]: No, most marine cyanobacteria are planktonic, some bloom-forming, e.g. *Trichodesmium*. Perhaps revise as "Most marine cyanobacteria of known public health significance grow along the shore…"?

A floating, visibly colored scum formed by floating cells may contain more than 10,000 cells/mL (Falconer, 1998). The floating scum, as in the case of *Microcystis* and *Anabaena spp*., may be concentrated by prevailing winds in certain surface water areas, especially at the shore. However, not all blooms result in scums floating on the surface since this depends on the buoyancy of the cells. For example, the gas vacuoles of *A. ovalisporum* and *C. raciborskii* act to regulate its position in the water column, not forming a floating scum, but concentrating with algal densities up to 100,000 cells/mL at several meters below the surface (ILS, 2000).

Depending on the nature of the cyanobacteria and the growth stage, cyanotoxins may be both within the cells (intracellular) and/or dissolved in the water (extracellular). In surface water, healthy bloom populations produce little extracellular toxin, with levels ranging from 0.1 to 10 µg/L (WHO, 1999). However, following the collapse of a large, highly toxic bloom, extracellular toxin concentrations can reach 1,800 µg/L or greater. In a healthy bloom, concentrations of intracellular cyanotoxins are several orders of magnitude higher than concentrations of the extracellular toxin (Rapala et al., 1997). Cylindrospermopsin, on the other hand, may be found at higher levels in solution than within cells (Rucker et al., 2007). The amount of toxin per cell or biovolume, the "toxin quota" (reported as µg toxin/g cyanobacteria), generally varies by a factor of two to five within the same taxon, although some species show greater variation (WHO, 1999).

On a dry-weight basis, the amount of toxin present is not necessarily proportional to the biomass (i.e., the number of cyanobacteria). The concentration of cyanobacterial toxins (µg toxin/g bacteria) in a small biomass of cyanobacteria with a high toxin quota could be higher than that of a large biomass of cyanobacteria with a low toxin quota, especially when the concentrations are reported on a dry-weight basis (WHO, 1999). However, these differences may not be as pronounced or may even be reversed if the concentrations of free toxin in water were reported on a volume basis (µg toxin/L water). In this case, the sheer mass of cyanobacteria with a low toxin quota could result in high toxin concentrations in the water.

In many cases, cyanotoxin data are expressed as milligrams or micrograms of toxin per gram dry weight (dw), with the dry weight measured by filtering and drying the water sample. As with concentrations reported per volume of water, there is a high degree of variation in cyanotoxin concentrations reported on a dry weight basis (WHO, 1999). If the data are obtained from cultures or bloom samples, the dry weight in such studies is principally cyanobacteria. If the samples are collected outside of scum areas, the dry weight also includes seston (particulate matter suspended in the water comprised of some cyanobacteria, other algae, zooplankton, and possibly soil and sediment). Therefore, the relative amount of cyanobacteria and seston affects the reported concentration of cyanotoxin per gram dry weight. In samples where the concentrations of seston were higher than the concentrations of cyanobacteria, the concentrations of cyanotoxin per gram total dry weight (cyanobacteria plus seston) were lower. Similarly, in samples where the concentrations of cyanobacteria were higher than the concentrations of seston, the concentrations of cyanotoxin per gram total dry weight (cyanobacteria plus seston) were higher. Because water samples contain both intracellular and extracellular cyanotoxins, concentrations of cyanotoxins reported in the literature as µg/L, rather than as concentration per dry weight (mg/g), usually refer to the total concentration of both the dissolved and intracellular toxin within a defined volume of water (WHO, 1999). Very high concentrations reported in the literature were taken from scums or dense accumulations of cyanobacteria. Therefore, based on the sampling location, there was a great deal of variation in the concentration data reported in the literature (as µg/L).

4.1.1 Surface Water

Freshwater blooms of hepatotoxic and neurotoxic cyanobacteria have been reported worldwide, becoming universally ubiquitous, with more blooms and toxic species being discovered (Codd, 1995; WHO, 2009). Surveys conducted in Florida, the Great Lakes and the midwest of the United States, and monitoring efforts in Ohio and Washington, indicate that freshwater cyanotoxins are prevalent in the U.S., mostly during warm seasons (Hudnell, 2010; Graham et al., 2010).

Commented [IS18]: Tautology here?

According to a survey conducted in Florida in 1999 between the months of June and November, the most frequently observed toxigenic cyanobacteria were *Microcystis, Cylindrospermopsis, and Anabaena spp*. (Burns, 2008). Of 167 surface water samples taken, 88% of these samples were positive for cyanotoxins. Microcystin was the most commonly found toxin in water samples collected (43%), occurring in all 87 bodies of water sampled. Cylindrospermopsin was detected in 34 water bodies (40% of samples) throughout the state and anatoxin-a was found in 29% of samples at concentrations up to 8 µg/L.

In 2002, the Monitoring and Event Response to Harmful Algal Blooms in the Lower Great Lakes (MERHAB-LGL) project collected water samples to create a data set on toxins, DNA, water quality and phycological analysis to evaluate the occurrence and distribution of cyanobacterial toxins in the lower Great Lakes region (Boyer, 2007). Microcystin was the most common toxin found with approximately 15% of the samples exceeding the WHO 1 µg/L guideline value and with detections in at least 65% of samples mostly in Lake Erie, Lake Ontario, and Lake Champlain. Anatoxin-a was the second most abundant toxin detected in 4% of the samples but at concentrations lower than 0.01 µg/L. Most of the blooms occurred during early summer (June-July) and anatoxin-a occurrence was suspected as the cause of an observed increase in the deaths of dogs and ducks during this period. Highly variable anatoxin-a concentrations were found in Lake Champlain, Lake Erie, and Lake Ontario ranging from more than 20 µg/L in 2003 and to no detection of cyanotoxins in 2002. The NOAA Center of Excellence for Great Lakes and Human Health (CEGLHH) continue monitoring the Great Lakes and regularly sample algal blooms for microcystin based on event response.

In 2006, the United States Geological Service (USGS) conducted a study of 23 lakes in the midwestern U.S. in which cyanobacterial blooms were sampled to determine the co-occurrence of toxins and taste-and-odor compounds in cyanobacterial blooms (Graham et al., 2010). The study showed that microcystin was detected in all the blooms, anatoxin-a was detected in 30% of the blooms, and cylindrospermopsin was detected in 9% of the blooms sampled. Mixtures of all the microcystin variants measured (LA, LF, LR, LW, LY, RR, and YR) were common, and all the variants were present in the blooms. Anatoxin-a was not found from communities dominated from producers like *Anabaena, Aphanizomenon, Planktothrix*, and *Pseudanabaena*. Although *Cylindrospermopsis* was detected in the study, the low concentrations of cylindrospermopsins occurred in communities dominated by *Aphanizomenon* or *Anabaena/Microcystis* and not by *Cylindrospermopsis*. The authors suggested that either *Cylindrospermopsis* strains in the U.S. do not produce the toxin as commonly produced elsewhere in the world, or the environmental conditions in the lakes sampled are not favorable for the toxic strains.

In 2005, Washington State Department of Ecology developed the Ecology Freshwater Algae Program to focus on the monitoring and management of blue-green algae in Washington lakes, ponds, and streams (WSTE, 2012). The data collected have been summarized in a series of reports for the Washington State Legislature (Hamel 2009, 2012). Microcystin levels ranged from the detection limit (0.05 µg/L) to 4,620 µg/L in 2008, 18,700 µg/L in 2009, 853 µg/L in 2010, and 26,400 µg/L in 2011. Anatoxin-a levels ranged from the detection limit to 144 µg/L in 2009, 538 µg/L in 2010, and 1,929 µg/L in 2011. Samples taken in 2010 for cylindrospermopsin were below the Washington State recreational guidance level of 4.5 µg/L and was not detected when sampled in 2011.

Since 2007, Ohio EPA (OHEPA) has been monitoring inland lakes for cyanotoxins, including Grand Lake St. Mary. In 2010, OHEPA sampled Grand Lake St. Mary for anatoxin-a, cylindrospermopsin, microcystin, and saxitoxin. Toxin levels ranged from below the detection limit to more than 2,000 µg/L for microcystin, to 15 µg/L for anatoxin-a, and to 9 µg/L for cylindrospermopsin. Follow on samples taken in 2011 for microcystin indicated concentrations more than 50 µg/L. During the same year, sampling in Lake Erie found microcystin levels to be more than 100 µg/L.

4.1.1.1 Microcystins

Microcystin is the most common cyanotoxin found worldwide. Concentrations ranging from 10 µg/L to 350 µg/L (up to 25,000 µg/L) have been reported in surface waters in most of the states of the U.S. and Europe (Funari and Testai, 2008). WHO (1999) has reported widely ranging concentrations of cyanobacterial toxins measured on a number of bases. Dry-weight concentrations of microcystin in surface freshwater cyanobacterial blooms or surface freshwater samples reported worldwide between 1985 and 1996 ranged from 1 to 7,300 µg/g. Water-volume concentrations of extracellular plus intracellular microcystin ranged from 0.04 to 25,000 µg/L. The concentration of dissolved, or extracellular, microcystin ranged from 0.02 to 200 µg/L. A high concentration of 1,800 µg/L extracellular microcystin was reported following treatment of a large bloom with algicide, which released the intracellular microcystin (WHO, 1999).

Cyanobacteria species that produce toxic microcystin such as *Microcystis*, *Anabaena*, *Nodularia*, *Aphanizomenon*, *Nostoc*, and *Oscillatoria* have been well characterized in temperate regions (Duy et al., 2000, Hawkins et al., 1997; Carmichael and Falconer, 1993). A 2004 Great Lakes study found high levels of cyanobacteria during the month of August (Makarewicz et al., 2006). Microcystin was detected at levels of 0.008 µg/L in the nearshore and 0.076 µg/L in the bays and rivers. Higher levels of microcystin, 1.6 to 10.7 µg/L, were found in smaller lakes in the Lake Ontario watershed.

In 2007, U.S. EPA and its state and tribal partners conducted a survey, the National Lakes Assessment (NLA), of the nation's lakes, ponds and reservoirs (U.S.EPA, 2009), the first-ever baseline study of the condition of the nation's lakes and provided unbiased estimates of the condition of natural and man-made freshwater lakes, ponds, and reservoirs greater than 10 acres and at least one meter deep. A total of 1,028 lakes were sampled for the NLA during summer 2007, representing the condition of about 50,000 lakes nationwide. The NLA looked at actual cyanobacterial cell counts and chlorophyll-a concentrations as indicators of the potential for the presence of algal toxins. It was the first-ever national study of algal toxins, specifically microcystin, in lakes. Microcystin samples were collected in open water at mid-lake, no samples were taken nearshore or other areas where scums were present.

The states with lakes with microcystin levels above the WHO's moderate risk threshold in recreational water (> 10 µg/L) are shown in Table 4-1. Microcystin was found to be present in about one third of lakes. According to results from the NLA, the concentration of microcystin in these samples ranged from the limit of detection (0.05 µg/L) to 225 µg/L. Of the 48 states sampled, two states, North Dakota and Nebraska, had 9% of the samples with detections of microcystin above the WHO guideline value for high risk for microcystin in recreational waters. Other states like Iowa, Texas, South Dakota, Utah, and Ohio also had samples that exceeded the moderate threshold value for recreational waters established by the WHO. In Ohio, 36% of the 19 selected lakes sampled during the NLA had detectable levels of microcystin (OHEPA, 2012). The highest microcystin concentration detected was 78 µg/L in Grand Lake St. Mary. U.S. EPA began a second survey in 2012 but data regarding algal toxins in the lakes sampled have not been published.

Table 4-1. States surveyed as part of the 2007 National Lakes Assessment with waterbody Microcystin concentrations above the WHO advisory guideline level for recreational water of 10 µg/L

State	Number of Sites Sampled	Percentage of Samples with Detection of Microcystin >10 µg/L	Minimum Detection of Microcystin >10 µg/L	Maximum Detection of Microcystin
North Dakota	38	9.1%	12 µg/L	192 µg/L
Nebraska	42	9.1%	18 µg/L	225 µg/L
South Dakota	40	4.9%	12 µg/L	33 µg/L
Ohio	21	4.5%	78 µg/L	78 µg/L
Iowa	20	4.5%	38 µg/L	38 µg/L
Utah	26	3.6%	15 µg/L	15 µg/L
Texas	51	1.8%	28 µg/L	28 µg/L

Microcystins have been detected in most of the states of the U.S. and over the years, many studies have been done to determine their occurrence in surface water. USGS, for example, did a study in the Upper Klamath Lake in Oregon in 2007 and detected microcystin concentrations between 1 µg/L and 17 µg/L (Vanderkooi et al, 2010). USGS also monitored Lake Houston in Texas from 2006 to 2008, and found microcystin in 16% of samples with concentrations less than or equal to 0.2 µg/L (Beussink and Graham, 2011). In 2011, USGS conducted a study on the upstream reservoirs of the Kansas River, a primary source of drinking water for residents in northeastern Kansas, to characterize the transport of cyanobacteria and associated compounds (Graham et al., 2012). Concentrations of total microcystin were low in the majority of the tributaries with the exception of Milford Lake, which had higher total concentrations detected, some exceeding the Kansas recreational guidance level of 20 µg/L. Upstream from Milford Lake, a cyanobacterial bloom was observed with total microcystin concentrations of 150,000 µg/L. When sampled a week later, total concentrations were less than 1 µg/L maybe due to dispersion of the bloom through the water column or to other areas, or a decline in the bloom due to settling of cyanobacteria out of the water column. Samples taken during the same time from outflow waters contained total microcystin concentrations of 6.2 µg/L, less than 1% of the concentration observed in the upstream bloom samples.

Other surveys and studies have also been conducted to determine the occurrence of microcystin in lakes in the United States. A survey conducted during the spring and summer seasons in 1999 and 2000 in more than 50 lakes in New Hampshire found measureable microcystin concentrations in all of the samples (Haney et al., 2000). In 2005 and 2006, a study conducted in New York waters, including Lake Ontario, found variability in microcystin-LR concentrations within the Lake Ontario ecosystem (Makarewicz et al., 2009). Of the samples taken in Lake Ontario coastal waters, only 0.3% of the samples exceeded the WHO provisional guideline value for drinking water of 1µg/L. However, 20.4% of the samples taken at upland lakes and ponds within the Lake Ontario watershed, some of them sources of drinking water,

exceeded the WHO guideline value for drinking water. During 2008 and 2009, a study was done in Kabetogama Lake, Minnesota to detect microcystin concentrations from algal blooms (Christensen et al., 2011). Microcystin concentrations were detected in 78% of bloom samples. Of these, 50% were above the WHO guideline of 1 µg/L in finished drinking water, and two samples were above the high risk WHO recreational level of 20 µg/L.

4.1.1.2 Anatoxin-a

Anatoxin-a has been found around the world and in the U.S. (Carmichael, 1997b). Dry-weight concentrations of anatoxin-a in surface freshwater cyanobacterial blooms or surface freshwater samples reported worldwide between 1985 and 1996 ranged from 0.4 to 4,400 µg/g. Water-volume concentrations of extracellular and intracellular anatoxin ranged from 0.02 to 0.36 µg/L (WHO, 1999).

4.1.1.3 Cylindrospermopsin

C. raciborskii is found in freshwater ponds, rivers, reservoirs, and eutrophic lakes and has been found in Australia, Asia, Europe, Africa, and South, Central, and North America (ILS, 2000, Fuentes 2010). Cylindrospermopsin-producing cyanobacteria occur in tropical or subtropical regions, but have also been detected in warmer temperate regions. In Florida, *C. raciborskii* was found to dominate one lake all year round (Burns, 2000). In 2006, *C. raciborskii* was detected in lakes in South Louisiana (Fuentes et al., 2010). The conditions identified in the study that promoted its growth were shallow and warm surface water (over 30°C), and low light intensities. The highest concentrations were observed from June until August with densities ranging from 37,000 cells/mL to more than 160,000 cells/mL. A study done in 2005 in two lakes connecting directly to Lake Michigan found low concentrations which were only observed in the late summer and with elevated bottom water temperatures and phosphorus concentrations (Hong et al., 2006).

In 2005, Oklahoma and the U.S. Army Corps of Engineers detected cylindrospermopsin at a maximum concentration of 1.6 µg/L (Lynch and Clyde, 2009). During the same year in Wisconsin, sixty-five samples were taken in Castle Rock and Petenwell lakes for blue-green algae and toxin identification (Evans, 2011). Cylindrospermopsis, a non-common algae in Wisconsin, was found in only 6% of the samples taken.

4.2 Occurrence in Drinking Water

In drinking water, the occurrence of cyanotoxins depends on their level in the raw source water and the effectiveness of treatment methods for removing cyanobacteria and cyanotoxins during the production of drinking water. Currently, there is no program in place to monitor for the occurrence of cyanotoxins in surface-water treatment plants and drinking water in the U.S. Therefore, data on the presence of cyanotoxins in drinking water and finished drinking water is scarce and generally not published. The occurrence information below was obtained from a

survey conducted by the American Water Works Association Research Foundation (AWWARF) and the published literature.

The AWWARF conducted the first study on the occurrence of algal toxins in raw and treated drinking waters in the United States and Canada in 1996 (Carmichael et al., 2001). Although in low concentrations, microcystin was found in waters sampled, including treated waters. No information on other toxins or toxin concentrations was available.

A survey conducted in 1999 in Florida (Burns, 2000) found that microcystin occurred in pre- and post-treated drinking water and was the most commonly found toxin. Finished water concentrations ranged from below detection levels to 12.5µg/L. Cylindrospermopsin was also detected in nine finished drinking water samples with concentrations from 8µg/L to 97µg/L. Anatoxin-a was found in three finished drinking water samples at concentrations up to 8µg/L.

A study from 2002 conducted during a dry summer growing season, evaluated the water quality and environmental parameters, including phytoplankton chlorophyll a concentrations, cyanobacterial assemblages, and microcystin concentrations in 11 potable water supply reservoirs within the North Carolina Piedmont (Touchette et al., 2007). The study found that cyanobacteria were the dominant phytoplankton community, averaging 65-95% of the total cells. The mean densities of the cyanobacteria species found, including *C. raciborskii*, were greater than 105cells/mL. Although microcystin concentrations were detected in nearly all samples, they were detected below the WHO provision guideline value of 1µg/L for MC-LR.

Concentrations exceeding the WHO provisional drinking water guideline value of 1µg/L have been detected during sampling undertaken in 2005 and 2006 in lakes and ponds used as a source of drinking water within the Lake Ontario watershed (Makarewicz et al., 2009). Microcystin-LR concentrations of 5.070µg/L were found in Conesus Lake, a source of public water supply that provides drinking water to approximately 15,000 people. Microcystin-LR was also detected a levels exceeding the WHO guideline value (10.716µg/L) in Silver Lake, a public drinking water supply for four municipalities.

4.3 Exposure in Water

Human exposure to cyanotoxins may occur by ingestion of toxin contaminated water or food, and by inhalation and dermal contact during bathing or showering and during recreational activities in waterbodies with the toxins. For the purpose of this document, we will focus on exposure by ingestion of drinking water contaminated with toxins and exposure by ingestion of water contaminated with toxins from exposure through fish consumption or recreational activities in freshwater sources.

4.3.1 Surface Water

Exposure to cyanobacteria and their toxins during recreational activities can be through direct contact, inhalation and/or ingestion. Exposures are usually not chronic with the exception of regions with extensive and persistent cyanobacterial blooms or when using untreated surface

waters where the exposures can be subacute and/or subchronic. Acute cyanobacteria associated symptoms are usually mild and do not require medical assistance. Skin irritations, allergic reactions or gastrointestinal illnesses could also be caused by other chemicals dissolved in water during cyanobacterial blooms, as well as by other risk factors like bacteria and/or viruses, and can be erroneously attributed to exposure to cyanobacterial metabolites (Stewart et al., 2006a). However, many studies have reported that ingestion of cyanobacterial toxins may induce vomiting, diarrhea, gastroenteritis and bloody urine (Puiseux-Dao and Edery, 2006). Chronic exposures may result in liver disease with necrosis and fibrosis and, at high doses, death can occur. Livestock and pets are potentially exposed to higher concentrations of cyanobacterial toxins than humans since they are more likely to consume scum and water directly from the toxic bloom. Children recreating close to the shore of lakes and beaches are also of potentially high risk from exposure to nearshore blooms.

Commented [IS19]: This chapter by Puiseaux-Dao and Edery should arguably not be viewed as an authoritative source. It's poorly referenced; I'm not aware of hematuria being reported by humans in the context of recreational exposure to cyanotoxins. I may of course be wrong on that count, but the citation of Puiseaux-Dao & Edery doesn't cut it, is my take…

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4.3.2 Drinking Water

Cyanotoxins can be dissolved in drinking water either by the breakdown of a cyanobacterial bloom or by cell lysis. Exposure can occur as some water treatment technologies are not designed for removal of these toxins. Exposure to high cyanotoxin concentrations in drinking water can result in acute/short term effects. Because children consume more water per unit body weight than do adults, children may potentially receive a higher dose.

5.0 OCCURRENCE AND EXPOSURE FROM MEDIA OTHER THAN WATER

5.1 Exposures from soil and edible plants

Cyanobacteria are very adaptable and have been found to colonize infertile substrates, such as volcanic ash and desert sand (Jaag, 1945; Dor and Danin, 1996; Metcalf, 2012). They have been found also in soil where they play a role in the functional cycling of nutrients. In soil, cyanobacteria occur on the surface or up to several centimeters in depth, on the surface of rocks, inhabit snow, tree trunks and other (Adhikary, 1996). They have also been reported in deeper soil layers and may be transported by the percolating water or burrowing animals. Some freshwater species are halotolerant (salt tolerant) and have been found in very saline environments such as salt works or salt marshes. Cyanobacterial cells can bioaccumulate in zooplankton (Watanabe et al., 1992) and as a result of grazing may settle out of the water column leading to an accumulation in the sediment. Cyanobacteria cells deposited in sediment are then subject to breakdown by sediment bacteria and protozoa thereby releasing their toxins.

Cyanobacteria form symbiotic relationships with plants and animals, such as fungi, bryophytes, pteriodophytes, gymnosperms and angiosperms (Rai, 1990). Cyanotoxins can be transmitted to food plants from surface irrigating waters when cyanotoxins are deposited on the plants' leaves. A study done with lettuce plants grown with spray irrigation with what was concentrated in water containing *M. aeruginosa*, detected levels ranging from 0.094 to 2.487 µg/g dw. These levels were detected in lettuce leaf extracts after 10 days from the last irrigation with water with microcystin-LR concentrations up to 3.23 µg/mg dw (Codd et al., 1999). In a study done by Chen in 2004, extracts from rape and rice seedlings were exposed to water with concentrations of microcystin-LR up to 3 mg/L (Chen et al., 2004a). The study found concentrations of microcystin-LR of 651ng/g in extracts from rape and 5.4ng/g in rice. These studies and others with high concentrations of cyanotoxins found that concentrations at these levels are able to inhibit plant growth causing visible toxic effects on the plant such as turning leaves brown. Studies with cyanotoxin concentrations typically found in natural surface waters (1-10 µg/L) showed that the toxins were only observed in the roots at levels of no concern to human health (Jarvenpa et al., 2007).

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Uptake of microcystins was measured in vegetable plants irrigated with contaminated groundwater in Saudi Arabia. Concentration of total MC was highly variable in the plants but positively correlated with concentrations in the wells. Radishes had the highest concentration of 1.2 µg/g fresh weight and cabbages had the lowest amount at 0.07 µg/g fresh weight; lettuce, parsley, arugula, and dill also had measurable concentrations. Generally, roots accumulated more than the leaves (Mohamed and Al Shehri, 2009).

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5.2 Exposures from fish and shellfish consumption

Cyanotoxins can bioaccumulate in common aquatic vertebrates and invertebrates, including fish, snails (Carbis et al., 1997; Beattie et al., 1998; Chen et al., 2005b; Berry et al., 2012), and mussels (Eriksson et al., 1989a; Falconer et al., 1992; Prepas et al., 1997; Watanabe et al., 1997; Funari and Testai, 2008). Exposure to cyanotoxins may occur following the

consumption of fish from reservoirs with blooms of toxin producing cyanobacteria. In 2008, U.S. EPA and California Agencies warned recreational users attending Iron Gate and Copco reservoirs on the Klamath River in Northern California to avoid contact with blue-green algae and eating fish caught in the reservoirs (CEPA, 2012). Algal toxins were detected in fish from Copco and Iron Gate reservoirs; however, the risk posed to human health by consuming fish is still being determined by the Office of Environmental Health Hazard Assessment (OEHHA), California Department of Public Health.

The health risk by consumption depends on the bioaccumulation of toxins in edible fish tissue. Microcystin has been shown to bioaccumulate in the liver and hepatopancreas (Williams et al., 1997) while cylindrospermopsin bioaccumulates in fish viscera and muscle tissue. Since fish are generally more tolerant of algal toxins than mammals, they tend to accumulate them over time (ILS, 2000).

Commented [IS27]: Maybe some further exposition required here? Hepatopancreas presumably refers to decapod crustacean tissues in this context, whereas the reader is led to think that this section is solely about fish? Also, MCs can also be found in fish muscle (many citations in the literature).

Commented [IS28]: Supporting citation needed here. I didn't think there was much in the literature on CYN bioaccumulation in fish, but I haven't done a recent search…

Levels of microcystins found in tissues of aquatic species potentially consumed by humans are shown in Table 5-1. Unless specified, levels are reported as MC-LR equivalents. Most studies have concentrated on levels in fish, although limited data show measurable amounts of MC-LR in mussels, shrimp, and crayfish. Recent reviews emphasize that MC levels in edible fish and shellfish are highly variable depending on trophic level, bloom conditions, and potential for depuration (Ibelings and Chorus, 2007; Ferrão-Filho and Kozlowsky-Suzuki, 2011). In fish, higher concentrations were consistently measured in liver compared with muscle, which is a significant dietary contribution in small fish consumed whole.

In a survey by Xie et al. (2005) MC-LR content in muscle was highest in carnivorous and omnivorous fish and was lowest in phytoplanktivorous and herbivorous fish. Chen et al. (2009) also found highest total MC levels in liver and muscle from omnivorous fish compared with other types of feeders. Berry et al. (2011) found the highest levels in phytoplanktivores and omnivores with no MC detected in predominantly zooplanktivore fish.

MC-LR was not detected in the livers from northern pike and white sucker fish collected from a lake in Canada following peak seasonal MC levels measured in the water (Kotak et al., 1996).

Table 5-1. Bioaccumulation studies of Microcystin in fish, shellfish, and crustaceans

Species/tissue	Tissue Concentration	Sampling Conditions	Average Water: Tissue Correlations	Reference
Fish				
Tilapia Muscle Liver Viscera	0.002-0.337 µg/g ww 0-31.1 µg/g ww 0-71.6 µg/g ww	3-year sampling from coastal lagoon; seston concentrations ranged from 0-980 µg/L during the study period	19.6 µg/L : 0.016 µg/g muscle 17 µg/L : 0.03 µg/g muscle 4.7 µg/L : 0.028 µg/g muscle	Magalhães et al., 2001
Tilapia Muscle Liver	0.007-0.06 µg/g 0.092-0.28 µg/g	Average levels from laboratory feeding of isolated cells	14.6 µg/fish/day (28 days) : 0.08 µg/g muscle (peak)	Soares et al., 2004
Fish - muscle	0.0396 µg/g ww	Peak level in samples from bay over 11 months	0.78 µg/L : 0.0396 µg/g muscle	Mahalhães et al., 2003
Carp (*Cyprinus carpio* L.) Muscle Hepatopancreas	0.038 µg/g fresh wt 0.261 µg/g fresh wt	Laboratory feeding bloom scum at 50 µg/kg body weight for 28 days	See previous columns	Li et al., 2004
Corydoras paleathus and *Jenynsia multidentata* Muscle Liver Gill	0.04-0.11 µg/g ww 1.62-19.63 µg/g ww 0.56-1.40 µg/g ww	Laboratory exposure to 50 µg MC-RR/L for 24 hours	See previous columns	Cazenave et al., 2005 (MC-RR)
Odontesthes bonariensis Muscle Liver Gill	(average/maximum) 0.05/0.34 µg/g ww 0.16/1.01 µg/g ww 0.03/0.10 µg/g ww	Wild caught from cyanobacteria containing reservoir; cellular MC-RR = 41.59 µg/g (wet season) and 9.65 µg/g (dry season)	Maximum tissue levels correlated to wet season	Cazenave et al., 2005 (MC-RR)
8 species Muscle Liver Intestine	1.81 µg/g dw 7.77 µg/g dw 22 µg/g dw	Wild caught from lake during bloom; 240 µg/g dry weight of bloom sample	Water not sampled; ingestion by fish possible.	Xie et al., 2005
Yellow perch Muscle Liver	0.00012-0.004 µg/g dw 0.017-1.182 µg/g dw	Wild caught from lake during summer months; 0.00016-4.28 µg/L in seston	Data presented graphically; positive correlation	Wilson et al., 2008

Species/tissue	Tissue Concentration	Sampling Conditions	Average Water: Tissue Correlations	Reference
4 species Muscle Liver	 0.002-0.027 µg/g dw 0.003-0.150 µg/g dw	Wild caught from lake during August; Total MC (-RR, -YR, -LR) in scum = 328 µg/g dry weight	See previous columns; tissue concentrations varied by species	Chen et al., 2009
Carassius gibelio Muscle Liver	(averages) 0.007 µg/g 0.124 µg/g	Wild caught from 7 lakes; 1.086-15.896 µg/L in scum; 0.21-3.749 µg/L in water	Individual data presented graphically	Papadimitriou et al., 2010
2 species Muscle Liver	 0.005-0.157 µg/g 0.094-0.867 µg/g	Commercial catch from lake with bloom; 0.02-0.36 µg/L in seston; 0.16-0.19 µg/L in water	Samples not matched to fish	Berry et al., 2011
Multiple Muscle Whole	 0.0005-1.917 µg/g ww 0.0045-0.215 µg/g ww	Multiple temperate and tropical lakes; 0.1-57.1 µg/L in water for all lakes	See paper, multiple fish samples from all lakes	Poste et al., 2011
3 species Muscle Liver	 <det. limit-0.32 µg/g <det. limit-0.27 µg/g	Wild caught in lake	Multiple samples from lake and fishes, highly variable	NDEQ, 2011
Shellfish				
Mussel - several species Whole body Foot/muscle	 0.064-0.188 µg/g ww 0.009-0.022 µg/g ww	Mean values from literature; water concentrations not given	Not possible	Ibelings and Chorus, 2007
Crustaceans				
Crayfish – whole (not found in muscle tissue)	2.9 µg/g dw	Experimental feeding for 11 days with *M. aeruginosa* isolated from a lake; MC content not measured	None possible	Vasconcelos et al., 2001
Crab – muscle	0.103 µg/g ww	Peak level in samples from bay over 11 months	0.78 µg/L : 0.103 µg/g	Mahalhães et al., 2003
Shrimp - several species Whole Muscle	 0.051-0.114 µg/g ww 0.004-0.006 µg/g ww	Mean values from literature; water concentrations not given	Not possible	Ibelings and Chorus, 2007

Very limited information was available on anatoxin-a accumulation in fish. Juvenile common carp, *Cyprinus carpio*, were exposed to freeze-dried cells of *Anabaena* sp. at a cell density of 10^5 or 10^7cells/mL for four days. Toxin content in whole fish was 0.005 and 0.073 µg/g fresh weight (Osswald et al., 2007).

Levels of cylindrospermopsin found in tissues of aquatic species potentially consumed by humans are shown in Table 5-2. One study determined the concentration of cylindrospermopsin in redclaw crayfish and rainbow fish sampled from aquaculture ponds. Cylindrospermopsin concentrations were 0.9 and 4.3 µg/g freeze-dried tissue in muscle and hepatopancreas, respectively, from crayfish and 1.2 µg/g freeze-dried tissue in the viscera from rainbow fish (Saker and Eaglesham, 1999). The study also showed that bioaccumulation could be greater in fish due to the longer exposure time in natural bodies of water to cyanobacterial blooms. Recent reviews also included levels of cylindrospermopsin in freshwater mussels and prawns (Kinnear, 2010; Funari and Testai, 2008; Ibelings and Chorus, 2007).

Table 5-2. Bioaccumulation studies of Cylindrospermopsin in fish, shellfish, and crustaceans

Species/tissue	Concentration	Conditions	Reference
Fish			
Rainbow fish - viscera	1.2 µg/g freeze dried tissue	Aquaculture pond during bloom; 589 µg CYN/L	Saker and Eaglesham, 1999
Shellfish			
Alathyria pertexta pertexta	0.13-0.56 µg/g fresh tissue	Experimental exposure to reservoir water; <0.8 µg/L	Kinnear, 2010
Swan mussel Hemolymph Viscera Whole body	 61.5 µg/g dry tissue 5.9 µg/g dry tissue 2.9 µg/g dry tissue	Experimental; 14-90 µg/L	Kinnear, 2010
Mussel Whole body Viscera	 0.247 µg/g wet wt. 1.099 µg/g wet wt.	Experimental exposure concentration not given; secondary citation	Funari and Testai, 2008
Crustaceans			
Crayfish muscle tissue hepatopancreas	0.9 µg/g freeze dried tissue 4.3 µg/g freeze dried tissue	Aquaculture pond during bloom; 589 µg CYN/L	Saker and Eaglesham, 1999
Prawns - flesh	0.205 µg/g wet wt.	Survey; CYN concentrations not given	Ibelings and Chorus, 2007

5.3 Exposures from use of dietary supplements

Extracts from *Spirulina* spp. and *Aphanizomenon flos-aquae* cyanobacteria have been used as dietary supplements known as bluegreen algae supplements (BGAS) (Funari and Testai,

Commented [IS29]: Are they extracts? I thought they were intact, lyophilized cellular material?

2008). These supplements are used to include support in losing weight during hypocaloric diets, and increasing alertness and energy and elevated mood for people suffering depression (Jensen et al., 2001). In children, they have been used as an alternative, natural therapy to treat attention deficit hyperactivity disorders (ADHD). Recent studies suggest contamination of BGAS with different levels of microcystin-LR ranging up to 35 µg/g and with some levels higher than 1 µg/g dry weight. These levels are probably from other toxin-producing cyanobacteria included during collection processes from the natural environment (Dietrich and Hoeger, 2005; Saker et al., 2005).

Commented [IS30]: Ponderous sentence structure

The death of a 34 year old woman due to liver failure was related to chronic consumption of BGAS (Dietrich et al., 2007). Although the cause and effect evidence has not been definitively established, the BGAS consumed by the patient contained 2.62 to 4.06 µg microcystin-LR equivalents/g dw and the patient's liver tissue tested positive for microcystin-LR after immunostaining.

Heussner et al. (2012) analyzed 18 commercially available BGAS for the presence of toxins. Neither anatoxin-a nor cylindrospermopsin were found in any of the supplements. However, all products containing *A. flos-aquae* tested positive for microcystins at levels ≤ 1 µg MC-LR equivalents/g dw. The microcystin contamination was primarily MC-LR with traces of MC-LA.

6.0 TOXICOKINETICS

6.1 Absorption

6.1.1 Microcystins

No data were available that quantified the intestinal, respiratory or dermal absorption of microcystin. Most of the available evidence indicates that absorption from the intestinal tract and into liver, brain, and other tissues requires facilitated transport using receptors belonging to the Organic Acid Transporter polypeptide (OATp) family. The OATp family transporters are part of a large family of membrane receptors that facilitate cellular, sodium-independent uptake and export of a wide variety of amphipathic compounds including bile salts, steroids, drugs, peptides and toxins (Cheng et al., 2005; Fischer et al., 2005).

For this document the abbreviation for the Organic Acid Transporter polypeptides will be written as OATp rather than differentiating the animal versions from the human versions by using lower case letters for the animals and upper case letters for humans.

Oral Exposure

Ito et al. (1997a) and Ito and Nagai (2000) qualitatively studied the oral absorption of MC-LR (purified from an algal bloom sample) following a single gavage dose of 500 µg/kg. Targeted immunostaining indicated that MC-LR was absorbed primarily in the small intestine, although some absorption did occur in the stomach (Ito and Nagai, 2000). Erosion of the surface epithelial cells of the small intestine villi was observed which might facilitate uptake of the toxin into the bloodstream (Ito and Nagai, 2000, 1997a).

The oral bioavailability of MC-LR was indirectly studied *in situ* using isolated intestinal loops of rats (Dahlem et al., 1989). Rats given an infusion of MC-LR (>95% pure) into the ileum showed clinical signs (i.e., labored breathing and circulatory shock) plus evidence of liver toxicity within 6 hours of a single 5 mg/kg dose. Infusion of a similar dose into a jejunal loop produced a lower degree of liver toxicity. These results suggest that there could be site-specificity in intestinal absorption of MC-LR; however, differences in absorptive surface area were not considered when proposing the hypothesis on differences in absorptive capacity.

Oral absorption of ^{3}H-dihydromicrocystin (75 µg/kg) was also demonstrated in swine using ileal loop exposure (Stotts et al., 1997a,b). In the exposed swine, the maximum blood concentration of the toxin occurred 90 minutes after dosing.

Inhalation Exposure

Microcystins are not volatile and are not likely to be present in gaseous form in air at ambient temperatures. However, they can be present as aerosols generated by wind and during showering or swimming thereby providing contact with the respiratory epithelium. Pulmonary absorption of MC-LR (purified from an algal bloom sample) was demonstrated by intratracheal instillation of sublethal dose of 50 µg/kg or a lethal dose of 100 µg/kg in mice (Ito et al., 2001).

Immunostaining of the lung occurred within 5 minutes. A lag period of 60 minutes occurred after the lethal dose and 7 hours after the sublethal dose before staining was observed in the liver. These data demonstrate that uptake from the lungs into systemic circulation can occur.

Dermal Exposure

No *in vivo* or *in vitro* studies of microcystin dermal absorption were identified.

Commented [IS31]: There is a report of lethal MC-LR intoxication by the dermal route, see citation #50 (Wannemacher et al 1987) in Stewart *et al* (2009) *Environ Health* 8:52. Although this report arguably has little relevance for considering the potential for water-soluble microcystins to cross an intact skin barrier under natural exposure conditions. The study of Wannemacher et al used DMSO as a vehicle, which is a highly artificial exposure.

6.1.2 Anatoxin-a

No quantitative data were located regarding the rate or extent of absorption of anatoxin-a in humans or animals. However, acute oral toxicity studies in animals indicate that anatoxin-a is rapidly absorbed as shown by the occurrence of clinical signs of neurotoxicity including loss of coordination, muscular twitching, and death from respiratory paralysis within several minutes of exposure (Fitzgeorge et al., 1994; Stevens and Krieger, 1991a).

6.1.3 Cylindrospermopsin

No quantitative data were located regarding the rate or extent of absorption of cylindrospermopsin in humans or animals following oral, inhalation or dermal exposure. Absorption of cylindrospermopsin from the gastrointestinal tract of mice is demonstrated by hepatic and other systemic effects observed in 14-day and 11-week oral toxicity studies of pure cylindrospermopsin (Humpage and Falconer, 2003; Shaw et al., 1999, 2000, 2001).

Filaments of *C. raciborskii* were found in the gut of animals that received oral doses of the cyanobacteria; however, it is not known whether cyanobacteria can survive or multiply in the gastrointestinal tract.

Commented [IS32]: This rumination is somewhat out of left field, is it not? Wouldn't a more pertinent question to pose in this context be along the lines of "The degree to which various cyanobacterial cells can remain intact through various stages of the G-I tract before lysis and release of intracellular cyanotoxins is poorly understood." ?

6.2 Distribution

6.2.1 Microcystins

The distribution of microcystins is limited due to the poor ability of these toxins to cross cell membranes (Puiseux-Dao and Edery, 2006). Facilitated transport is apparently necessary for both uptake of microcystins into organs and tissues as well as for their export. Many studies have demonstrated that inhibition of microcystin uptake by its OATp transporter reduces or eliminates the liver toxicity observed following *in vitro* or *in vivo* exposures (Runnegar et al., 1981, 1995a; Runnegar and Falconer, 1982; Hermansky et al., 1990a,b; Thompson and Pace, 1992). OATps are located in the liver, brain, testes, lungs, kidneys, placenta and other tissues of rodents and humans (Cheng et al., 2005). Only a few of the OATps have been characterized at their functional, structural, and regulatory levels. In mice, males often express OATps in tissues to a greater extent than females (Cheng et al., 2005).

Commented [IS33]: See earlier comment re validity of the Puiseaux-Dao & Edery chapter as an authoritative reference. The opening phrase here "The distribution of microcystins is limited…" reads a bit odd to me. Isn't distribution across various organs and tissues a relative concept, pertaining to toxicokinetics? The uptake may be limited, but the distribution?

Commented [IS34]: Is this right? Isn't this sentence as written contradicted by the following paragraph on reduced toxicity in OATP knockouts?

Human OATp1B1, OATp1B3, and OATp1A2 were shown to mediate the transport of ^{3}H-dihydroMC-LR in *Xenopus laevis* oocytes, and its uptake was inhibited by sulfobromophthalein and taurocholate (Fischer et al., 2005). Additionally, a number of *in vitro* studies have indicated that cells lacking microcystin-competent OATp do not absorb microcystin and that introduction of OATps to these cells enables them to absorb microcystin (Jasionek et al., 2010, Komatsu et al., 2007, Feurstein et al., 2010, Fischer et al., 2010). One study indicated that the role of OATp in microcystin uptake varies by congener, with greater uptake of MC-LW and MC-LF than of MC-LR and MC-RR (Fischer et al., 2010).

Lu et al. (2008) used OATp1b2 null mice to demonstrate the importance of the OATp system for transporting MC-LR into the liver. OATp1b2 knockout mice did not experience the severe hepatotoxicity and death caused by 120 µg MC-LR/kg (i.p.) in wild-type mice. Fischer et al. (2010) demonstrated the necessity for microcystin-competent OATp for transport of microcystin across the cellular membrane using primary human hepatocytes and OATp-transfected HEK293 cells compared to control vector HEK293 cells, which were resistant to microcystin cytotoxicity. Primary human hepatocytes were an order of magnitude more sensitive than the OATp-transfected HEK293 cells. The study authors suggested that this was due to the fact that the transfected HEK293 cells only contained OATp1b1 and 1b3, while the primary human hepatocytes may contain other OATps that contribute to the uptake of the microcystin congeners. Komatsu et al. (2007) observed similar results, but also found that MC-LR accumulation in OATp-transfected HEK293 cells increased in a dose-dependent manner, which was not observed in the control vector HEK293 cells.

Oral Exposure

The distribution of MC-LR (purified from an algal bloom sample) following oral gavage administration to mice (500 µg/kg) was investigated using immunostaining methods (Ito and Nagai, 2000). MC-LR was detected in large amounts in the villi of the small intestine. Erosion of the villi was observed, which may have enhanced absorption of the toxin into the bloodstream. MC-LR was also present in the blood plasma, liver, lungs, and kidneys.

Once inside the cell, these toxins covalently bind to cytosolic proteins, resulting in their retention in the liver. The hepatic cytosolic proteins that bind microcystin have been identified as the protein phosphatase enzymes PP1 and PP2A. Covalent adducts of MC-LR, MC-LA, and MC-LL with both enzymes were identified by reverse-phase liquid chromatography. In contrast, the dihydromicrocystin-LA analog did not form covalent bonds with PP1 and PP2A which suggests a role for the double bonds of Adda in the covalent binding. However, the dihydromicrocystin analog was able to inhibit the enzyme activity supporting a role for electrostatic interactions in the mode of action (MOA) for enzyme inhibition as well as covalent binding; the IC_{50} was similar for MC-LR and the dihydro-analog (Craig et al., 1996).

The distribution of ^{3}H-dihydroMC-LR in mice was shown to differ for the oral and i.p. injection routes of exposure (Nishiwaki et al., 1994). Intraperitoneal (i.p.) injection of ^{3}H-dihydroMC-LR resulted in rapid and continuous uptake by the liver, with approximately 72% of the administered dose present in the liver after 1 hour. Small amounts of radiolabel were found in the small intestine (1.4%), kidney and gallbladder (0.5%), lungs (0.4%) and stomach (0.3%).

Oral administration of ^{3}H-dihydroMC-LR resulted in much lower concentrations in the liver, with less than 1% of the administered dose found in the liver at either 6 hours or 6 days post administration, and about 38% of the dose found in the gastrointestinal contents.

The distribution of ^{3}H-MC-LR (>95% pure) was evaluated following i.p. injection of a sublethal (45 µg/kg) or lethal (101 µg/kg) dose in mice (Robinson et al., 1989). The tissue distribution of radiolabel was similar after injection of either the lethal or sublethal dose. Liver accumulation reached a maximal value of 60% by 60 minutes. For the 101 µg/kg dose, the liver, intestine and kidney contained 56, 7 and 0.9% of the radiolabel, respectively. Heart, spleen, lung and skeletal muscle each contained less than 1% of the radiolabel. Within one minute of a sublethal i.v. injection (35 µg/kg) in mice, MC-LR was distributed to the liver, kidneys, intestines, carcass (body minus the heart, lung, liver, gut, kidney, and spleen), and plasma (Robinson et al., 1991). After 60 minutes, the liver contained about 67% of the dose, which did not change across the 6 days of the study. Sixty minutes after the i.v. exposure the intestines had 8.6%, the carcass had 6%, the kidneys had 0.8%, and only trace amounts were left in the plasma. Levels in the lung were highest within 3 minutes with none detectable within 10 minutes. There was measurable radiolabel in the spleen.

MC-LR was not found in the milk of dairy cattle that were exposed to *M. aeruginosa* cells via drinking water (Orr et al., 2001; limit of detection 2 ng/L) or ingestion of gelatin capsule containing the cells (Feitz et al., 2002; limit of detection 0.2 ng/L).

Inhalation Exposure

Immunostaining methods were used to evaluate the organ distribution following intratracheal instillation of MC-LR purified from an algal bloom sample (Ito et al., 2001). Following instillation of a lethal dose (100 µg/kg), the lung, liver, small intestine and kidney were positively stained for MC-LR. Intense staining was observed in the lung by 5 minutes post-instillation, followed by the kidney (10 minutes), the small intestine (45 minutes) and the liver (60 minutes). After approximately 90 minutes, bleeding began around the hepatic central vein. The authors described the pathological changes in the liver as essentially the same as those seen following oral or i.p. injection exposure routes. Intratracheal instillation of a sublethal dose (50µg/kg) resulted in immunostaining of the lung, liver, kidney, cecum and large intestine (Ito et al., 2001). No discernible pathological changes were observed at this dose level.

Other Exposure Routes

Data from humans accidentally exposed to microcystin from dialysis water indicates that a large proportion of microcystin in the serum and liver is bound to protein (Yuan et al., 2006). Three methods were compared to detect microcystin in stored sera and liver samples from the exposed dialysis patients: 1) direct competitive enzyme-linked immunosorbent assay (ELISA) using a polyclonal antibody against microcystin, which detects free microcystin in a supernatant fraction; 2) liquid chromatography-mass spectrometry (LC/MS) after oxidation and solid phase extraction to detect bound microcystin in a protein pellet fraction; and 3) gas-chromatography-MS (GC/MS) after oxidation and solid phase extraction to detect total microcystin in a sera or liver homogenate. The GC/MS method found a higher concentration of microcystin compared

to ELISA and LC/MS which was hypothesized to be due to better detection of the covalently bound form of microcystin.

Tissue distribution was evaluated in mice given i.v. injection of a sublethal dose of ^{3}H-MC-LR by Robinson et al. (1991). The liver contained approximately 67% of the radiolabel by 60 minutes, and the amount of hepatic radioactivity did not change throughout the 6-day study period, despite urinary and fecal elimination of 24% of the administered dose. The subcellular distribution of radioactivity in the liver demonstrated that approximately 70% of the hepatic radiolabel was present in the cytosol. *In vitro* experiments showed that radiolabeled microcystin in the liver was bound to high molecular weight cytosolic proteins (Robinson et al., 1991). The nature of the binding was demonstrated to be covalent, saturable and specific for a protein with a molecular weight of approximately 40,000. Binding was inhibited by okadaic acid (a potent inhibitor of serine/threonine phosphatases [1 and 2A]), suggesting that the target protein is protein phosphatase 1 or 2A. Binding proteins for MC-LR were found in cytosol derived from several different organs, suggesting that liver specificity is not due to limited distribution of target proteins. Covalent binding to hepatic proteins may be responsible for the long retention of microcystin in the liver.

Brooks and Codd (1987) reported extensive liver uptake following i.p. injection of 125 µg/kg of a ^{14}C-labelled toxin extracted from *M. aeruginosa* strain 7820 (assumed to be a microcystin) in mice. Seventy percent of the radiolabel was found in the liver after 1 minute, increasing to almost 90% after 3 hours. Radiolabel was also found in the lungs, kidneys, heart, large intestine, ileum and spleen.

Lin and Chu (1994) evaluated the kinetics of MC-LR distribution in serum and liver cytosol derived from 24 mice. Uptake of pure MC-LR into the serum, as analyzed by direct competitive ELISA was shown to be rapid following an i.p. injection of 35µg/kg (sublethal dose). The toxin reached a maximum concentration in the serum by 2 hours and in liver cytosol by 12 hours post-injection. MC-LR was shown to be bound to liver cytosolic proteins and the kinetics of binding was correlated with inhibition of protein phosphatase 2A activity. The maximum decrease in enzyme activity was observed 6-12 hours following injection.

The organ distribution of a ^{125}I-labeled heptapeptide toxin (MW 1019) isolated from *M. aeruginosa* was investigated in female rats following i.v. administration of 2 µg of the peptide (Falconer et al., 1986; Runnegar et al., 1986). The heptapeptide toxin was purified by HPLC prior to reaction with ^{125}I in the presence of NaI and lactoperoxidase. The highest tissue concentrations of labeled peptide were detected in the liver and kidney. After 30 minutes, 21.7% of the administered dose was present in the liver, 5.6% was present in the kidneys, 7% remained in the gut contents, and 0.9% was cleared in the urine (Falconer et al., 1986). The authors reported that no significant accumulation was observed in other organs or tissues.

Liver Tissues – in vitro

Most of the early research on the toxicity of microcystins identified the liver as the most severely impacted organ. As a result, many researchers have examined the distribution to the liver using cell cultures. Pace et al. (1991) demonstrated significant accumulation of ^{3}H-MC-LR

in isolated perfused liver despite a low overall extraction ratio (16% in liver, 79% in perfusate). In the liver, radiolabel corresponding to MC-LR (15%) and a more polar metabolite (85%) was primarily found in the cytosolic fraction.

The cellular uptake of ^{3}H-dihydroMC-LR was evaluated using primary rat hepatocytes in suspension and in isolated perfused rat liver (Eriksson et al., 1990a; Hooser et al., 1991a). The uptake (as measured by scintillation counting of washed cells) of a mixture of unlabeled MC-LR and ^{3}H-dihydroMC-LR was shown to be specific for freshly isolated rat hepatocytes (Eriksson et al., 1990a). The uptake of ^{3}H-dihydroMC-LR was shown to be inhibited by bile acid transport inhibitors such as antamanide, sulfobromophthalein and rifampicin, and by the bile salts cholate and taurocholate.

Hooser et al. (1991a) found that the uptake of ^{3}H-dihydroMC-LR was rapid for the first 5-10 minutes, followed by a plateau, in both rat hepatocyte suspensions (four replicates; two from each of two rats) and the isolated perfused rat liver (n = 2). Uptake was measured as radioactivity in fractionated cells versus radioactivity in medium. The uptake of ^{3}H-dihydroMCLR was inhibited by incubation of suspended rat hepatocytes at 0°C, suggesting the involvement of an energy-dependent process. Uptake was also inhibited by preincubation of hepatocytes with rifampicin, a competitive inhibition of the bile acid transporter (member of OATp family).

Runnegar et al. (1991) studied the influence of dose level and exposure time on the uptake of ^{125}I- MC-YM in isolated rat hepatocytes (measured as radioactivity in centrifuged cell pellet). Hepatocyte uptake was initially rapid with a plateau in the uptake rate observed after 10 minutes. The initial uptake rate (in the first minute of exposure) increased with increasing concentration, but cumulative uptake ceased at a dose that resulted in plasma membrane blebbing.

MC-YM uptake by isolated rat hepatocytes, as measured by cell associated radioactivity and assays for protein phosphatase inhibition in cell lysates, was temperature-dependent and was inhibited approximately 20-60% by *in vitro* preincubation with bile acids or bile acid transport inhibitors (taurocholate, trypan blue, cholate, sulfobromophthalein, cyclosporine A, trypan red and rifamycin) (Runnegar et al., 1995a). This provides evidence to indicate that microcystin uptake occurs by carrier mediated transport. Pretreatment with protein phosphatase inhibitors (i.e., okadiac acid and calyculin A) resulted in the inhibition of both MC-YM uptake and protein phosphatase inhibition, suggesting that the OATp is itself regulated by serine/threonine phosphorylation of protein phosphatases.

Primary cultures of liver cells cease to express these OATps after 2-3 days of being maintained in culture. Therefore, established liver cell lines are generally not useful for evaluating microcystin toxicity (Eriksson and Golman, 1993; Heinze et al., 2001). Chong et al. (2000) evaluated microcystin toxicity in eight permanent cell lines (including rodent, primate and human cell lines), only two of which (human oral epidermoid carcinoma KB cells and rat Reuber H35 hepatoma H-4-II-E cells) showed cytotoxicity following MC-LR exposure. The toxic response in these cells was most evident if MC-LR was added when the cells were seeded. Established monolayers were more resistant to microcystin toxicity.

The subcellular distribution of ^{3}H-dihydroMC-LR was evaluated using primary rat hepatocytes in suspension and the isolated perfused rat liver (Hooser et al., 1991a). ^{3}H-dihydro LR was primarily localized in the cytosolic fraction in both the hepatocytes and liver. In the hepatocytes, protein precipitation with trichloroacetic acid indicated that approximately 50% of the ^{3}H-dihydroMC-LR was found as free toxin, while the remaining 50% was bound to cytosolic proteins. Since little of the radiolabel was in the insoluble pellet containing insoluble actin and other elements, the authors suggested that ^{3}H-dihydroMC-LR did not bind significantly to actin or other cytoskeletal proteins (Hooser et al., 1991a).

The subcellular protein binding of ^{3}H-dihydroMC-LR was evaluated in rat liver homogenates (Toivola et al., 1994). Most of the radiolabeled toxin (80%) was bound to cytosolic proteins. ^{3}H-dihydroMC-LR was shown to bind both protein phosphatase 1 (PP1) and protein phosphatase 2A (PP2A); however, PP2A was detected primarily in the cytosol, while PP1 was found in the mitochondrial and post-mitochondrial particulate fraction (membrane proteins).

6.2.2 Anatoxin-a

No information regarding the tissue distribution of anatoxin-a was identified in the materials reviewed for this assessment. However, anatoxin-a inhibits acetylcholine esterase and can act as a natural organophosphate insecticide (Puiseux-Dao and Edery, 2006). The rapid appearance of symptoms following exposure is consistent with rapid uptake from the gastrointestinal tract and serum distribution. In a study by Fitzgeorge et al (1994) deaths occurred within 2 minutes of gavage administration of doses greater than 5 mg/kg a as a result of respiratory paralysis.

Commented [IS35]: No. It's anatoxin-a(S) that operates as an anticholinesterase…

Commented [IS36]: Again, it's anatoxin-a(S) that is structurally a natural organophosphate. Not sure why the reference to "insecticide" in this context. Is anyone seriously suggesting that antx-a(S) actually be used as a pestricide? I'd have thought this observation is more of a structural chemistry factoid. And the Puiseaux-Dao & Edery citation rears its head yet again…

6.2.3 Cylindrospermopsin

No information was located regarding the tissue distribution of cylindrospermopsin following oral, inhalation or dermal exposure. The distribution and elimination of intraperitoneally administered ^{14}C-cylindrospermopsin (>95% pure; extracted and purified from lyophilized *C. raciborskii* cells) in normal saline was studied in male Quackenbush mice in a series of experiments using sublethal and lethal dose levels of the chemical (Norris et al., 2001). Analysis of liver, kidneys and spleen at 48 hours showed mean ^{14}C recovery of 13.1% of the dose in the liver and <1% in the other tissues. Total recovery of radiolabel from tissues and excreta was 85-90% of the administered dose in each of the four mice.

In a second experiment reported by Norris et al. (2001) 12 mice were administered a single 0.2 mg/kg dose of ^{14}C-cylindrospermopsin, which is the approximate median lethal i.p. dose (Norris et al., 2001). ^{14}C content was determined in the urine and feces in all animals after 12 and 24 hours, and in the liver, kidneys and spleen in five mice that were euthanized after 5-6 days due to toxicity (effects not specified) and after 7 days in the surviving 7 mice that had no signs of toxicity. The overall mean (standard deviation) recoveries of ^{14}C in the liver, kidneys and spleen after 5-7 days were 2.1 ± 2.1, 0.15 ± 0.14, and <0.1% (no standard deviation

Commented [IS37]: This is the 5-day mouse i.p. LD50. Probably important to state that here, as there is an order of magnitude difference between the 24-hr and the 5-day LD50 doses.

provided) of the dose, respectively. The broad standard deviations are indicative of considerable inter-individual differences in response. Comparison of data from four mice with signs of toxicity and four mice without signs of toxicity showed no clear relationship between toxicity and patterns of tissue distribution, although there was a trend toward decreased liver retention in the surviving mice.

A third experiment, in which excretion and tissue distribution were assessed in four mice that were given a 0.2 mg/kg i.p. dose of ^{14}C-cylindrospermopsin and evaluated after 6 hours was also reported by Norris et al. (2001). Label was detected in all tissues that were examined (liver, kidney, heart, lung, spleen, blood and bile), but occurred predominantly in the liver (20.6% [range: 14.6-27.9] and kidneys 4.3% [range: 3.7-4.7] of the dose, respectively. A week after dosing, about 2% of the label was still detectable in the liver.

Uptake of purified cylindrospermopsin into a kidney cell line (Vero cells) was found to be slow and progressive, and not energy dependent (Froscio et al., 2009). The cylindrospermopsin-induced effects on cellular protein synthesis in the Vero cells could not be reversed when the toxin was removed. The strong interaction of the toxin with its target to inhibit protein synthesis indicates cylindrospermopsin remains in the intracellular environment for extended periods. Although dilution of the cell media increased the uptake of cylindrospermopsin, no mechanism could be identified that explained this result.

6.3 Metabolism

6.3.1 Microcystins

Limited data are available on the metabolism of microcystins. Most of the studies discussed below indicate that there is minimal if any catabolism. The microcystins can be conjugated with glutathione and cysteine to increase their solubility and facilitate excretion (Kondo et al., 1996; Ito et al 2005a). It is not clear whether CYP450-facilitated oxidation precedes conjugation. ^{3}H-dihydroMC-LR is not extensively metabolized in swine liver after i.v. injection or ileal loop exposure, and is primarily present in hepatic tissues as the parent compound (Stotts et al., 1997a,b).

Some metabolism of MC-LR was shown to occur in mice and in isolated perfused rat liver (Robinson et al., 1991; Pace et al., 1991). Male CD-1 mice were administered ^{3}H-MC-LR as an i.v. dose of 35 µg/kg and monitored for up to six days. Over the 6-day interval, 9.2% and 14.5% of the dose was excreted in the urine and feces, respectively, of which ~60% was parent compound. High-performance liquid chromatography analysis for urinary and fecal metabolites revealed several minor peaks of lower retention times. Analysis of liver cytosol preparations revealed that 83% of the radiolabel was bound to a high molecular weight cytosolic protein after six hours and that amount decreased to 42% by day 6 (Robinson et al., 1991). In isolated perfused rat liver, binding of both the parent toxin (^{3}H-MC-LR) and a more polar metabolite to cytosolic proteins was also demonstrated by Pace et al. (1991). Polar metabolites accounted for 65-85% of the hepatic cytosol radiolabel. Metabolites of MC-LR were not further characterized in these studies.

Administration of 125 µg/kg of *Microcystis* toxin 7820 to mice resulted in decreased amounts of cytochrome b5 and cytochrome P450 in the liver (Brooks and Codd, 1987). Pretreatment of mice with microsomal enzyme (mixed function oxidase) inducers (β-naphthoflavone, 3-methylcholanthrene and phenobarbital) was shown to eliminate this effect on hepatic cytochromes and to extend survival and reduce liver toxicity (i.e., changes in liver weight). However, no change in cytochrome P450 associated enzyme activity (i.e., metabolism of aminopyrene and p-nitrophenol) was found in microsomes isolated from mouse liver after animals were injected with an extract of *M. aeruginosa* (Cote et al., 1986).

Glutathione and cysteine conjugates have been identified in the liver after i.p. injection of 10 or 20 µg MC-RR to mice or 4µg MC-LR to rats (purified from blooms) (Kondo et al., 1992, 1996). The conjugates were isolated and compared to chemically prepared standards which indicated structural modification of the Adda and Mdha moieties of the microcystin toxins. The authors postulated that these moieties could be the sites of CYP oxidation and subsequent conjugation with glutathione or cysteine.

Glutathione conjugates of MC-LR are formed by glutathione S-transferase (GST) enzymes found in both rat liver cytosol and microsomes (Takenaka, 2001). Glutathione conjugation of LR (> 95% pure isolated from *M. aeruginosa*) by five recombinant human GSTs (A1-1. A3-3, M1-1, P101, and T1-1) has been characterized (Buratti et al., 2011). All five GSTs catalyzed the conjugation, but with different dose-responses. The study report also determined that the spontaneous reaction for MC-LR conjugation with GSH was dependent on GSH concentration, pH, and temperature.

Glutathione and cysteine conjugates of MC-LR and MC-YR were demonstrated to be less toxic than the parent compounds based on LD_{50} estimates, but were still significantly toxic (LD_{50} values ranged from 217 to 630 µg/kg in mice) (Kondo et al., 1992). Metcalf et al. (2000) also demonstrated that microcystin conjugates with glutathione, cysteine-glycine and cysteine were less toxic in the mouse bioassay than the parent compounds; these conjugates were also shown to be weaker inhibitors of protein phosphatases 1 and 2A *in vitro*. Glutathione and cysteine conjugates of MC-LR were primarily distributed to the kidney and intestine following intratracheal instillation in mice (Ito et al., 2002a). This result suggests that the lower toxicity of glutathione and cysteine conjugates may be related to distribution to excretory organs and elimination of metabolites *in vivo*.

Ito et al. (2002b) synthesized glutathione and cysteine conjugates of MC-LR and administered them by intratracheal instillation in mice. The metabolites were demonstrated to be less toxic than the parent compound as shown by lethal doses about 12-fold higher than the LR lethal dose. The metabolites were distributed primarily to the kidney and intestine, as opposed to the liver (Ito et al., 2002b).

6.3.2 Anatoxin-a

No information regarding the metabolism of anatoxin-a was identified in the materials reviewed for this assessment.

6.3.3 Cylindrospermopsin

There is evidence indicating that the hepatic CYP450 enzyme system is involved in the metabolism and toxicity of cylindrospermopsin. Pretreatment of hepatocytes with known inhibitors of CYP450 (50 µm proadifen or ketoconazole) diminished the *in vitro* cytotoxicity of cylindrospermopsin (Froscio et al., 2003). Similarly, pretreatment of mice with the CYP450 inhibitor piperonyl butoxide protected against the acute lethality of cylindrospermopsin in male Quackenbush mice (Norris et al., 2002). Support for the involvement of the CYPs is provided by Shaw et al (2000, 2001) who demonstrated that a main target of cylindrospermopsin toxicity is the periacinar region of the liver, an area where substantial CYP450-mediated xenobiotic metabolism occurs.

Runnegar et al., (1995a) investigated the decrease in cellular GSH and its role in the metabolism and toxicity of cylindrospermopsin in primary cultures of rat hepatocytes. To ascertain whether the fall in GSH was due to decreased GSH synthesis or increased GSH consumption, total GSH was measured after treatment with 5 mM buthionine sulfoximine (BSO, an irreversible inhibitor of GSH synthesis). The rates of fall in total GSH (nmol/10^6 cells/hr) were 8.2 (±2.5), 6.0 (±1.7), and 5.9 (±1.3) for control, 2.5 µM and 5 µM cylindrospermopsin pretreated cells, respectively. This suggests that the fall in GSH induced by the toxin was due to the inhibition of GSH synthesis rather than increased consumption, because in the latter case the rate of fall in GSH would have been accelerated by toxin pretreatment. Furthermore, excess GSH precursor (20 mM N-acetylcysteine), which supported GSH synthesis in control cells, did not prevent the fall in GSH or toxicity induced by cylindrospermopsin. Addition of CYP450 inhibitors α-naphthoflavone, SKF525A and cimetidine partially prevented the decrease in cell GSH inducted by cylindrospermopsin. Results suggest the formation of an oxidized and possibly glutathione conjugated derivative which may be a more potent inhibitor of GSH synthesis than the parent cylindrospermopsin.

^{14}C-cylindrospermopsin (>95% pure; extracted and purified from lyophilized *C. raciborskii* cells) was studied in a series of mouse experiments that utilized the i.p. route of exposure (Norris et al., 2001). A single dose of 0.1 mg/kg was administered to 4 male Quackenbush mice (6 weeks old) or 0.2 mg/kg to12 mice. Urine and fecal samples were collected and weights obtained at 12 hour intervals after dosing. The 4 mice were sacrificed 48 hours after dosing. Urine, fecal, liver and kidney samples were extracted with methanol to precipitate proteins, and the ^{14}C in the supernatant was fractionated using high performance liquid chromatography (HPLC) for the detection of metabolites. No attempt was made to fractionate or otherwise identify the ^{14}C in the protein precipitate.

Analysis of methanol extracts of urine samples suggested that a large part (72%) of the excreted ^{14}C was present as cylindrospermopsin. Some (~23.5%) of the urinary ^{14}C was detected in protein precipitated by the methanol, suggesting the presence of a protein-bound metabolite. The authors did not indicate whether the level of protein in the urine was normal or abnormal. Analysis of liver tissue showed the presence of ^{14}C in both a methanol extract and protein precipitate. When fractionated by HPLC, the methanol extracted ^{14}C from the liver had the same elution characteristics seen in some of the urine methanol extracts, suggesting the presence of the same metabolite. No methanol-extractable metabolite was found in kidney tissue.

The authors could not rule out the possibility that the non-extractable ^{14}C in the liver was protein-bound cylindrospermopsin, although the evidence from Runnegar et al. (1995a) and Shaw et al. (2000) suggested that it might be a metabolite. Runnegar and Shaw provided evidence of the need for activation of cylindrospermopsin for toxicity suggesting the presence of one or more metabolites. Although, no identification of metabolites was performed, results indicate the metabolite is either more polar than cylindrospermopsin or that the parent compound is fragmented during metabolism.

6.4 Excretion

6.4.1 Microcystins

Limited information on the elimination of microcystin from the human body is available from follow-up of dialysis patients exposed unintentionally to microcystin in dialysis water. More than 50 days after documented exposure, microcystins were detected in patients' serum by ELISA using polyclonal antibodies against MC-LR with cross reactivity against several microcystin analogues (Hilborn et al., 2007; Soares et al., 2006).

Biliary excretion has been shown in both *in vivo* and *in vitro* studies. Falconer et al. (1986) administered an i.v. dose of 2 µg microcystin in saline extracted from *M. aeruginosa* to female albino rats. After 120 minutes, 9.4% of the administered dose was present in the intestinal contents and 1.9% was present in the urine, suggesting that biliary excretion plays a significant role in elimination. Similarly in isolated perfused rat liver, 1.7% of radiolabeled MC-LR was recovered in the bile by the end of the 60-minute perfusion (Pace et al., (1991). In the bile collected during the perfusion, 78% of the radiolabel was associated with the parent toxin, while the remaining radiolabel was associated with more polar metabolites.

In a study by Robinson et al. (1991), male VAF/plus CD-1 mice were administered an i.v. dose of 35 µg/kg of radiolabeled MC-LR. A total of approximately 24% of the administered dose was eliminated in the urine (9%) and feces (15%) during the 6-day study monitoring period. Approximately 60% of the excreted microcystin, measured at 6 and 12 hours following injection, was present in the urine and feces as the parent compound.

Elimination in swine was evaluated following i.v. injection or ileal loop exposure (Stotts et al., 1997). ^{3}H-dihydroMC-LR was detected in the bile as early as 30 minutes after i.v. injection of 75µg/kg. Following ileal loop exposure to the same dose, the concentration of toxin was consistently higher in the portal venous blood as compared to peripheral blood. This suggests that first pass metabolism may play a role in the clearance of MC-LR.

6.4.2 Anatoxin-a

No information regarding the elimination of anatoxin-a was identified in the materials reviewed.

6.4.3 Cylindrospermopsin

No information was located regarding the elimination of cylindrospermopsin following oral, inhalation or dermal exposure. The elimination of i.p administered ^{14}C-cylindrospermopsin (>95% pure; extracted and purified from lyophilized *C. raciborskii* cells) in saline was studied in male Quackenbush mice in a series of experiments using sublethal and lethal dose levels of the chemical (Norris et al., 2001).

In one experiment, four mice were given a single sublethal dose of 0.1 mg/kg, and urine and feces were collected at 12 hour intervals for the following 48 hours. The mean cumulative excretion of ^{14}C in the first 12 hours after dosing was 62.8% of the administered dose in the urine and 15.5% in the feces. There was little additional excretion of ^{14}C in either the urine or feces following 12 additional hours. The 15.5% mean fecal excretion value reflects a very high fecal excretion in one of the four animals (nearly 60% of the dose compared to <5% in the other mice); the authors considered the possibility that the high value in the one animal resulted from the injection entering the upper gastrointestinal tract, but concluded that this possibility was unlikely given the injection technique used. Total mean recovery in the urine, feces, liver, kidneys and spleen was 85-90% of the ^{14}C dose in each of the four mice.

The second experiment reported by Norris et al. (2001) included 12 mice administered a single 0.2 mg/kg dose of ^{14}C-cylindrospermopsin, which is the approximate median lethal i.p. dose (Norris et al., 2001). ^{14}C content was determined in the urine and feces in all animals after 12 and 24 hours. Results were similar to those obtained with a sublethal dose (reported above), except that there was some continued urinary and fecal excretion over the second 12 hours of the monitoring period. The mean cumulative urinary and fecal excretion of ^{14}C was 66.0 and 5.7% of the dose within 12 hours, and 68.4 and 8.5% of the dose within 24 hours, respectively. The mean total 25 recovery in the urine and feces after 24 hours was 76.9% of the administered dose. The overall mean recoveries of ^{14}C in the liver, kidneys and spleen after 5-7 days were 2.1, 0.15 and <0.1% of the administered dose, respectively. Comparison of data from four mice with signs of toxicity and four mice without signs of toxicity showed no clear relationship between toxicity and patterns of excretion, although trends toward increased urinary excretion and decreased fecal excretion in surviving mice were suggested.

Commented [IS38]: 5-day LD50 (see earlier comment)

In a third experiment, four mice were given a 0.2 mg/kg i.p. dose of ^{14}C-cylindrospermopsin and tissue distribution (urine, feces, blood, gall bladder, liver, heart, kidney, lung and spleen) was evaluated after 6 hours (Norris et al., 2001). The mean cumulative urinary and fecal excretion of ^{14}C after 6 hours was 48.2 and 11.9% of the administered dose, respectively. One of the four mice indicated more than 40% of the dose in the feces (additional data and data on metabolites not reported).

6.5 Pharmacokinetic Considerations

6.5.1 Microcystins

The blood half-life was measured following i.v. administration of a ^{125}I-labelled heptapeptide toxin extracted from *M. aeruginosa* (MW 1019, assumed to be a microcystin) (Falconer et al., 1986). A biphasic blood elimination curve was demonstrated, with the first component having a half-life of 2.1 minutes and the second component having a half-life of 42 minutes.

MC-LR excretion was also evaluated in mice (Robinson et al., 1991). A biexponential plasma elimination curve was observed following i.v. injection of a sublethal dose of 35 µg/kg of ^{3}H –MC-LR. Plasma half-lives of 0.8 and 6.9 minutes were reported for the first and second phase of elimination, respectively.

Stotts et al. (1997a,b) evaluated the toxicokinetics of ^{3}H-dihydroMC-LR in swine following i.v. injection and ileal loop exposure. Elimination of labeled MC-LR was rapid and followed a biphasic pattern, suggesting that the liver rapidly removes the toxin from the blood. Clearance from the blood is slower at higher dose levels, presumably due to the liver toxicity and circulatory shock observed at high doses. It is important to take into consideration that tritium radiolabeling may alter the MC molecule's ability to bind with protein phosphatases, thus altering the MC protein binding and tissue distribution profile (Hilborn, et al. 2007).

No physiologically based toxicokinetic models have been developed for microcystins.

6.5.2 Anatoxin-a

No data on half-life or other quantitative pharmacokinetic data applicable to anatoxin-a were identified.

6.5.3 Cylindrospermopsin

No data on half-life or other quantitative pharmacokinetic data applicable to cylindrospermopsin were identified.

7.0 HAZARD IDENTIFICATION

Toxicological data on the adverse effects of anatoxin-a and cylindrospermopsin are relatively sparse when compared to the database for the microcystins. The topics covered within the sections in this chapter include human case reports and epidemiology data, reports of standard toxicological studies as well as genotoxicity and mechanistic data, and the characterization of hazard.

Table 7-1 provides a summary of the case reports and epidemiology studies on exposure to cyanotoxins during recreational activities. Cases of non-lethal human poisonings predominantly manifested as acute gastrointestinal disorders (e.g., nausea, vomiting and diarrhea). Other symptoms such as headache and muscle weakness were also reported. A number of these cases were documented by the detection of *Anabaena*, either alone or with *Microcystis*, in the feces. Deaths have been reported after exposure to anatoxin-a while swimming, and after intravenous exposure to microcystins in a dialysis clinic.

Commented [IS39]: Table 7.1 isn't just about recreational exposure to drinking water exposures. It also includes epidemiological investigations and outbreak studies of drinking water exposures and dialysis exposures. Table 7.1 is also a selected list of epidemiological and case investigations, there are plenty of gaps here. On what basis were the studies in table 7.1 selected for inclusion?

Anatoxin-a was implicated in the death of a 17-year-old boy who died 2 days after swallowing water while swimming in a pond containing an algal bloom (Behm, 2003). The boy went into shock and suffered a seizure before dying from heart failure. A companion teenage boy who also swallowed some of the pond water while swimming later became sick with severe diarrhea and abdominal pain but survived. Three other teenage boys who swam in the pond at the same time as other two, but had not been fully submerged in the water, developed only unspecified minor symptoms. Tests of stool samples from the two affected boys revealed the presence of *A. flos-aquae* cells (Behm, 2003). Results of initial analyses of liver, blood and ocular (vitreous) fluid samples from the boy who died indicated the presence of anatoxin-a but were negative for other cyanobacterial toxins (microcystins, cylindrospermopsins and saxitoxins) (Carmichael et al., 2001 and 2004). The coroner concluded that anatoxin-a was the most reasonable cause of the death based on the available information, but a definitive diagnosis was confounded by the delay between exposure and overt toxicity and the lack of other anatoxin-a - related human fatalities for a temporal comparison (Behm, 2003). In particular, the time of death is inconsistent with what is known about anatoxin-a toxicity as determined from laboratory animal studies (i.e., that signs of neurotoxicity and death typically occur within minutes to several hours of exposure). More recent (unpublished) analyses determined that the compound detected in the body fluids and liver tissue samples was not anatoxin-a but the amino acid D-phenylalanine (Carmichael et al., 2001 and 2004), obscuring the diagnosis.

Commented [IS40]: This citation predates the event under discussion, which occurred in 2002

In February 1996, there was an outbreak of acute liver failure in patients at a renal dialysis clinic in Caruaru, Brazil (Carmichael et al., 2001). One hundred and sixteen of 131 patients who received their routine hemodialysis treatment at that time experienced headache, eye pain, blurred vision, nausea and vomiting. Subsequently, 100 of the affected patients developed acute liver failure and, of these, 76 died. Analysis of the carbon, sand, and cation/anion exchange resin from in-house filters in the clinic's water treatment for microcystins and cylindrospermopsin demonstrated the presence of both cyanotoxins. Analyses of blood, sera, and liver samples from the patients revealed microcystins, but not cylindrospermopsin. However, the method used to extract cylindrospermopsin from the samples may have been inadequate. Based on a comparison of victims' symptoms and liver pathology to the data from

Table 7-1. Human Case Reports and Epidemiology Studies for Microcystin, Anatoxin-a, and Cylindrospermopsin Exposure

Putative agent and source	Location	Number of individuals and exposure scenario	Symptoms	Reference
Case Reports				
Cyanobacteria from lake water; *Microcystis* and *Anabaena* from stool samples	Saskatchewan, Canada; various lakes	Number not stated; recreational activities	Nausea, stomach pain, diarrhea, headache, muscle weakness	Dillenberg and Dehnel, 1960
Anabaena identified in one of the lakes	Pennsylvania, USA; two lakes	Number not stated; swimming	Headache, abdominal cramping, nausea, vomiting, diarrhea, hay fever-like symptoms, ear aches, eye irritation, sore throat, sneezing, runny nose, swollen lips	Billings, 1981
M. aeruginosa primarily in bloom; MC-LR identified	Staffordshire, England; reservoir	18 army recruits and soldiers; canoe exercises	Malaise, sore throat, headache, abdominal pain, dry cough, diarrhea, vomiting, blistered mouth; pneumonia in two 16-year old recruits	Turner et al., 1990
M. wesenbergii and *M. aeruginosa*; MC-LR identified	Salto Grande Dam, Argentina	19-year old male; immersed for 2 hours	Nausea, abdominal pain, fever, respiratory distress, dyspnea, transient liver and kidney damage	Giannuzzi et al., 2011
Anabaena and *Microcystis* cells identified	Itaparica Dam, Bahia, Brazil	1,118 cases; 70% children <5 years; exposure not characterized	Diarrhea cases coincided with flooding of reservoir	Teixeira et al., 1993
Anabaena, *Aphanizomenon*, *Planktothrix* identified	Murray River, Australia	102 gastrointestinal cases; 86 dermatological cases; use of untreated river water for domestic purposes	GI: abdominal pain, vomiting, diarrhea Dermal: rash, itching, blistering of mouth	el Saadi and Cameron, 1993; el Saadi et al., 1995
M. aeruginosa bloom	Malpas Dam reservoir, Australia	Number not stated; public water supply	Increased serum GGT levels	Falconer et al., 1983

Commented [IS41]: Many, indeed most of the studies cited in Table 7.1 did not confirm exposures to individual cyanotoxins, they were studies of exposure to (presumably) toxic cyanobacteria.

Anabaena and *Microcystis*	Not stated	Number not stated; ingestion of lake water	Nausea, vomiting, diarrhea	Schwimmer and Schwimmer, 1968
A. Flos-aquae bloom	Not stated	5 teenage boys; ingestion, swimming in pond	1 died, 1 severe diarrhea and abdominal pain after swallowing pond water; 3 minor symptoms did not get fully submerged	Behm, 2003
Microcystin and Cylindrospermopsin	Caruaru, Brazil	131 patients; contaminated dialysis water from water treatment filters in clinic	116 with headache, eye pain, blurred vision, nausea, vomiting; 100 acute liver failure; 76 died	Carmichael et al., 2001
Population Surveys and Epidemiology Studies				
Microcystin	Three Gorges Reservoir Region, China	1322 children; survey to correlate liver damage with levels in drinking water and aquatic food	Microcystin associated with increased AST, ALP; no change in ALT or GGT	Li et al., 2011a
Cyanobacteria cell counts	Australia	~338 individuals; use of water recreation sites	GI and dermal symptoms correlated with cell counts from water samples	Pilotto et al., 1997
Cyanobacteria cell counts	Australia	32,700 births; maternal drinking water	Increased incidence of low birth weight with increasing cyanobacteria exposure	Pilotto et al., 1999
Microcystin	Zhejiang Provence, China	408 cases of colorectal cancer; drinking water source	Positive association between microcystin exposure and colorectal cancer	Zhou et al., 2002
Microcystin	Haimen City, China	Number not stated, liver cancer; drinking water source	No association	Yu et al., 2002
Cyanobacteria	Florida, USA	Number not stated, liver and colorectal cancers; drinking water source	Possible risk of liver cancer and surface water service; no association for colorectal cancer and water source	Fleming et al., 2002; 2004

animal studies of microcystins and cylindrospermopsin, it was concluded that the major contributing factor to death of the dialysis patients was intravenous exposure to microcystins.

7.1 Microcystins

7.1.1 Human Effects

7.1.1.1 Epidemiological Studies

Zhou et al. (2002) conducted a retrospective cohort analysis of colorectal cancer and exposure to microcystins in drinking water in a Chinese province in which an association had been reported previously (Jiao et al., 1985; Chen et al., 1994). A total of 408 cases of primary colorectal adenocarcinoma diagnosed between 1977 and 1996 from eight randomly selected towns within Haining City of Zhejiang Province were used as the study population. Cases were identified using the local cancer registry and independently verified by two pathologists. Drinking water source used during the lifetime was used as a surrogate measure of exposure to microcystins. Information on drinking water source was obtained by interview of patients or family members of deceased cases. In each of the eight towns, 10 water sources (3 rivers, 3 ponds, 2 wells and 2 taps) were randomly selected and sampled for microcystins twice in each of the months of June through September (total of eight samples from each source). Water samples were analyzed for microcystins by ELISA; the authors did not specify the targeted congeners. The authors do not specify the nature of the "tap" water sources, but the text implies that the tap water derived from one or more treatment plants.

The average incidence rate of colorectal cancer across all of the study areas was 8.37/100,000 per year. The incidence rate was compared among the four different water sources, with well water users serving as the referent population. Compared with the incidence among well water users, the colorectal cancer incidence rates among users of the other water sources were significantly increased. Tap water use was associated with a relative risk (RR) of 1.88, while river and pond water use were both associated with a relative risk over 7.0. There was no difference in colorectal cancer incidence between river and pond water users. The authors suggested that exposure to trihalomethane compounds might account for the increase in incidence among tap water users. Table 7-2 shows the incidence rate, relative risk and 95% CIs for these exposure comparisons.

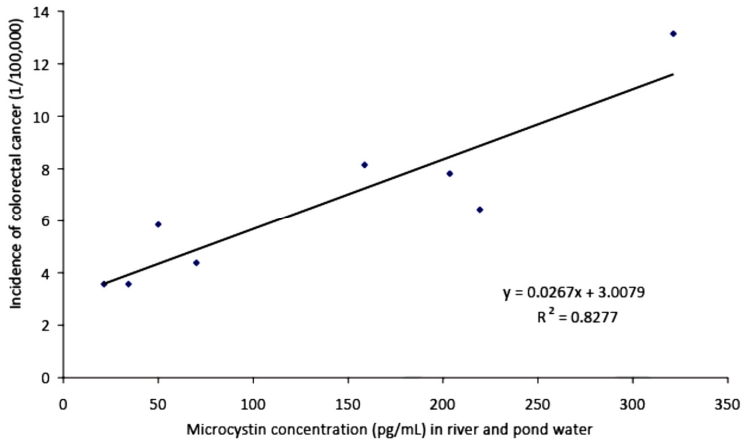
Table 7- 2. Relative Risk of Colorectal Cancer and Microcystin Concentration by Drinking Water Source

Water Source	Colorectal Cancer Incidence Rate per 100,000	Relative Risk of Colorectal Cancer	95% CI	Number of Microcystin Samples > 50 pg/mL	Mean Microcystin Concentration (pg/mL)	Maximum Microcystin Concentration (pg/mL)
Well water	3.61	-	-	0/12	0.73	9.13
Tap water	6.77	1.88	1.39-2.54	0/17	4.85	11.34
River water	28.5	7.94	6.11-10.31	25/69	141.08	1083.43
Pond water	27.76	7.7	5.75-10.30	6/35	106.19	1937.94

From Zhou et al., 2002

Microcystins were detected at concentrations exceeding 50 pg/mL (considered by the authors to be the limit for positive detection) only in river and pond water, and the average concentrations in these sources were substantially higher (30- to 50-fold) than well or tap water. A similar proportion (about 25%) of the residents in each of the eight towns used river and pond water for drinking water, allowing an analysis comparing the average microcystin concentration in river and pond water in each town with the incidence rate by town. This analysis showed a strong correlation between incidence rate and concentration of microcystin (Spearman correlation coefficient = 0.88, $p<0.01$). Figure 7-1 shows the relationship between colorectal cancer incidence and average microcystin concentration.

This study provides suggestive evidence for an association between microcystin exposure and colorectal cancer. It is also consistent with earlier reports of an association between drinking river or pond water and incidence of colorectal cancer in the Zhejiang Province of China (Jiao et al., 1985; Chen et al., 1994). Because demographic information was not provided in the report, it is not clear whether dietary, genetic, and lifestyle factors associated with colorectal cancer have been adequately controlled in the analysis. Further, other potential biological and chemical contaminants in the river and pond water, except trihalomethanes, were not considered.



From Zhou et al., 2002

Figure 7- 1. Relationship between Colorectal Cancer and Microcystin Concentration in River and Pond Water in Haining City, China

A number of epidemiological studies have been conducted in an area of Southeast China with high rates of hepatocellular carcinoma. These studies are summarized by IARC (2010) and Health Canada (2002). Overall a positive association was found between the risk for hepatocellular carcinoma and water source from surface waters. In an analysis of pooled data from six case-control studies, RR was 1.59 (confidence limits not given); estimates of RR from other individual studies ranged from 1.5-4 (IARC, 2010). Consumption of pond or ditch water was associated with a higher risk of liver cancer incidence when compared with well water consumption. Confounders such as hepatitis B infection and aflatoxin exposure were not generally considered in most studies. The presence of cyanobacteria in the water source was not a component of the study. Thus, the only relationship between these estimates of risk is the fact that cyanobacteria are primarily surface water contaminants.

Ueno et al. (1996) conducted a survey of microcystin content in drinking water supplies in Haimen City to test the hypothesis that microcystins in surface drinking water supplies could contribute to the higher incidence of liver cancer. Microcystins were measured by ELISA in shallow and deep wells, as well as in ponds/ditches and river waters. Occurrence of microcystins was higher in pond/ditch water (17% reported as positive with concentration >50 pg/mL) and river water (32% positive) samples than in shallow wells (4% positive) or deep wells (no detections >50 pg/mL). Further, microcystin concentrations averaged across the drinking water types were different, averaging 101, 160, and 68 pg/mL in pond/ditch, river, and shallow well samples, respectively. These data, while suggestive, do not directly associate exposure to microcystins and liver cancer, since individual exposures were not measured or estimated, and other biological or chemical contaminants in the surface waters have not been ruled out.

In a case-control study of liver cancer in Haimen City, China, a variety of liver cancer risk factors were evaluated, including hepatitis B and C virus infection, aflatoxin B1 or microcystin exposure, smoking, drinking, diet and genetic polymorphisms (Yu et al., 2002). From a pool of 248 patients with hepatocellular carcinoma and 248 age-, sex- and residence-matched controls, 134 paired cases and controls assented to blood samples for virus infection and ALDH2 and CYP2E1 gene polymorphism analyses. Data from these analyses were combined with questionnaire information on possible lifestyle and dietary risk factors for liver cancer. Microcystin exposure was assessed categorically based on drinking water supplied from tap, deep or shallow well, river, ditch, or pond water. Neither univariate nor multivariate analysis of the data indicated an association between consumption of river, pond, or ditch water and hepatocellular carcinoma. Hepatitis B virus infection was strongly associated with primary liver cancer, and history of i.v. injection was also identified as a risk factor (Yu et al., 2002).

Fleming et al. (2002) conducted an ecological epidemiological investigation of the relationship between drinking water source and incidence of primary liver cancer in Florida. The study was prompted by data showing cyanobacteria and toxins, especially microcystins, in surface drinking water sources in Florida. The study population consisted of all cases of primary hepatocellular carcinoma reported to the Florida state cancer registry between 1981 and 1988. The study population was divided into comparison groups consisting of those served by surface drinking water supply and those using other sources. Residence at the time of diagnosis was used to place cases into the various comparison groups. Surface water treatment plants and their service areas were geocoded, as were deep groundwater treatment plants. Several comparisons were made. First, incident cases residing in the service area of a surface water treatment plant were compared with those residing in the service area of a deep groundwater treatment plant. Within this comparison, there were several referent groups; one randomly sampled from the available groundwater service areas, one matched on median income and rent, one matched on ethnic makeup and one matched on income, rent and ethnicity. Second, incident cases in the surface water service area were compared with equally-sized buffer areas surrounding the surface water service area, but not served by the treatment plant. Finally, incident cases were compared with the incidence in the general Florida population.

Evaluation of the individual incidence rates in the 18 surface water service areas with the groundwater service areas did not reveal any statistically significant differences among the individual incidence rates. When the service areas were pooled, residence in a surface water service area was associated with a statistically significant reduced risk of hepatocellular carcinoma compared with either groundwater service areas (standardized rate ratios [SRR] ranged from 0.8 to 0.98 for the four groundwater comparison groups) or the general Florida population (SRR of 0.8). It should be noted that the measure of exposure, residence within a surface water service area, was estimated as the average size plus two standard deviations of the service area for this comparison.

When comparisons were made between residence in the actual (i.e., not estimated as above) surface water service areas and residence in the buffer areas surrounding the service areas, a statistically significant increase in the incidence of hepatocellular carcinoma was observed for those residing within the surface water service area (SRR=1.39, CI=1.38-1.4). Analyses of 1990 census data suggested that the ethnic and socioeconomic backgrounds of the

service areas and buffer areas were similar, although the authors did not report these data. Interestingly, the incidence of hepatocellular carcinoma in the buffer areas was significantly lower than that in the general Florida population (SRR=0.59).

An ecological study is useful for generating hypotheses, but not for establishing an exposure-response relationship due to the lack of exposure data on individuals. In this case, there is strong potential for misclassification of exposure. Residence in a surface water service area at the time of diagnosis of hepatocellular carcinoma is a poor measure of potential exposure to cyanobacterial toxins, especially given residential mobility and likely latency time for cancer development. Further, the initial comparisons with groundwater service areas used GIS-generated estimates of surface water service areas rather than actual service areas, leading to greater potential for misclassification.

Fleming et al. (2004) also conducted an ecological study assessing the relationship between incidence of colorectal cancer and proximity to a surface drinking water treatment plant, with the latter representing a surrogate for exposure to cyanobacteria. Methods for this study were identical to those described above for Fleming et al. (2002) except that colorectal cancer data were abstracted from the Florida Cancer Data System from 1981-1999. As with Fleming et al. (2002), comparisons were made between the colorectal cancer incidence rates in the 18 surface water treatment service areas with several referent groups (a random group of groundwater treatment service areas, a group of groundwater treatment service areas matched on median income and rent, a group of groundwater treatment service areas matched on ethnic makeup, a group of groundwater treatment service areas matched on both median income and ethnicity, groups residing in an equally-sized buffer areas surrounding the surface water service area and, finally, the general Florida population). Mann Whitney rank sum tests of all comparisons did not suggest an association between colorectal cancer and residence at time of diagnosis in a surface water treatment area (details not provided). This ecological study is subject to the same limitations as described above for Fleming et al. (2002).

7.1.2 Animal Studies

7.1.2.1 Acute Toxicity

Oral Exposure

Fawell et al. (1999a) studied the impact of single oral gavage doses of microcystin-LR (commercial product; purity not specified) in aqueous solution in male and female CR1:CD-1(ICR)BR(VAF plus) mice and CR1:CD(SD)BR(VAF plus) rats (five per sex per species). Doses of 500, 1,580 and 5,000 µg/kg body weight were administered. Untreated control groups were not included. The animals were observed for up to 14 days prior to sacrifice and necropsy. Microscopic examinations of the lung and liver were conducted. LD_{50} values were estimated as 5,000 µg/kg for mice and >5,000 µg/kg for rats.

Animals that died showed clinical signs, including hypoactivity and piloerection. However, clinical signs were absent in survivors. Body weight among surviving animals was not affected during the 14-day follow-up. Necropsy of the animals that died showed darkly

discolored and distended livers, as well as pallid kidneys, spleen, and adrenals. Livers of all animals that died had moderate or marked centrilobular hemorrhage. Diffuse hemorrhage in the liver was seen in rats and mice of all dose groups, but the incidence was not clearly related to dose. The incidence and severity of liver lesions increased in a dose-dependent fashion, as shown in Table 7-3.

Table 7- 3. Incidence of Liver Lesions in Mice and Rats Treated with a Single Dose of Microcystin-LR

Dose (µg/kg)	Number Animals Treated	Mortality	Diffuse Hemorrhage	Moderate Centrilobular Hemorrhage	Marked Centrilobular Hemorrhage	Centrilobular Necrosis	Cytoplasmic Vacuolation
Mice							
500	10	0	2	0	0	0	0
1580	10	1	1	2	1	0	0
5000	10	5	1	7	0	2	0
Rats							
500	10	0	8	0	0	0	0
1580	10	0	7	0	0	0	0
5000	10	1	8	1	1	1	1

From Fawell et al., 1999a

Yoshida et al. (1997) assessed the acute oral toxicity of purified microcystin-LR (>95% pure by HPLC) in female BALB/c mice. Preliminary experiments using doses of 16.8 and 20 mg/kg resulted in death within 160 minutes in two mice; therefore, doses of 0, 8.0, 10.0 and 12.5 mg/kg were chosen for LD_{50} determination. Microcystin-LR in saline solution was administered via gavage to a total of seven 6-week-old mice. Mortality within 24 hours was 0/2 controls, 0/1 at 8 mg/kg, 0/2 at 10 mg/kg and 2/2 at 12.5 mg/kg. The oral LD_{50} was identified as 10.9 mg/kg. The liver, kidneys and lung were sectioned and examined by light microscopy. Electron microscopy was used to identify apoptotic cells in the livers of treated mice. The remaining tissues were subjected to histopathological analysis.

No effects on the stomach, intestine, skin or organs other than the liver and kidneys were observed. Liver effects in animals that died included centrilobular hemorrhage and hepatocyte degeneration, as well as free hepatocytes in the veins of mice administered doses greater than 12.5 mg/kg (in the preliminary experiments). Effects on the kidneys included proteinaceous eosinophilic materials in the Bowman's spaces of mice receiving the preliminary doses of 16.8 and 20 mg/kg.

One of the surviving mice at 10.0 mg/kg was sacrificed after 24 hours. Evidence of hepatocellular necrosis was observed in the centrilobular and midzonal regions, and single cell death (possibly apoptotic) was reported in the centrilobular region, as well as surrounding necrotic areas. In the other mouse treated with 10 mg/kg and the two mice treated with 8.0 mg/kg (all sacrificed 1 week after treatment), the livers contained hypertrophic hepatocytes in the centrilobular region and fibrosis in the centrilobular and midzonal regions. A few apoptotic cells were observed in these animals. No kidney effects were reported in animals that survived treatment for at least 24 hours.

Fitzgeorge et al. (1994) administered microcystin-LR via gavage to newly weaned CBA/BALBc mice. The commercially-obtained compound was described only as "suitably purified." The LD_{50} was estimated to be 3,000 µg/kg, and increases in liver (43%) and kidney (5.9%) weights were reported. The authors reported that there was no change in lung or spleen weight; dose-response data and other endpoints were either not examined or not reported.

Ito et al. (1997a) compared the acute effects of microcystin-LR on the livers and gastrointestinal tracts of young and aged mice. Single doses of 500 µg/kg of microcystin-LR (purity not specified) dissolved in ethanol and diluted in saline were administered via oral gavage to aged (29 mice age 32 weeks) and young (12 mice age 5 weeks) male ICR mice. Three aged and three young untreated mice served as controls. Twenty-two aged mice were sacrificed at 2 hours, five mice at 5 hours, and two mice at 19 hours after treatment; four young mice were sacrificed at each time point. Liver damage and gastrointestinal erosion were evaluated.

The results showed that the effects in the aged mice were more severe than those in the young mice. In young mice, no liver pathology or gastrointestinal changes were reported. In contrast, 18 of 29 aged mice treated with the same dose developed pathological changes of the liver. Among the aged mice, 8 of 29 had liver injury of the highest severity, characterized as bleeding, disappearance of many hepatocytes in the whole liver and friable tissue (severity rating of +4). Five of 29 mice had liver changes characterized by bleeding and disappearance of hepatocytes in centrilobular region (severity rating of +3). Necrosis in the centrilobular region was observed in 4 of 29 mice (severity rating of +2) and eosinophilic changes in the centrilobular region were indicated for one mouse (severity rating of +1).

Gastrointestinal effects observed in the treated aged mice included necrosis to one-third depth of the mucosa and severe duodenal damage (including decreased villi density, separation of epithelial cells from lamina propria and edema of both the submucosa and villi). Details of the incidence of these effects were not reported; however, the authors indicated that the degree of liver injury was related to the severity of gastrointestinal effects. Regeneration of intestinal tissues was evident in some of the mice sacrificed at later time points (5 and 19 hours after treatment). Among untreated aged mice, serum enzyme levels (AST and ALT) were not different. Aged mice showed thinning of gastrointestinal epithelial cells with consequent exposure of lamina propria and glands in some areas.

Rao et al. (2005) compared the acute oral effects of microcystin extract in aged (36 weeks old) and young (6 weeks old) male Swiss albino mice. The extract was prepared from laboratory cultures of *M. aeruginosa* and the microcystin-LR content determined by HPLC. A single dose was administered of 3.5 g extract/kg which corresponded to 9.625 mg microcystin-LR/kg. Mortality first occurred after 4-5 hours with the mean time to death significantly shorter in the aged mice. Both groups of mice had increased relative liver weight and DNA fragmentation compared to control, but there was no difference between the age groups. In contrast, glutathione depletion and lipid peroxidation were significantly greater in the aged mice when compared with young mice. Further, while most serum enzymes were increased over controls in both groups, GGT was increased to a greater extent in aged mice than in young mice.

Inhalation Exposure

No studies of acute inhalation exposures were identified. The microcystins are not volatile; therefore inhalation exposures are likely to only occur in the form of aerosols. A brief abstract describes a study of acute microcystin-LR exposure via inhalation (Creasia, 1990). Details of study design and results were not reported. The LC_{50} for mice exposed to a microcystin-LR aerosol (nose only) for 10 minutes was reported to be 18µg/L (mg/m^3) with a 95% confidence interval of 15.0-22.0µg/L (mg/m^3). Based on studies of lung deposition after exposure of mice to the LC_{50} concentration, an LD_{50} of 43 µg/kg body weight was estimated. The authors reported that histological lesions in mice killed by aerosol exposure were similar to those in mice dosed intravenously with MC-LR.

Several studies demonstrated the potential for uptake from the respiratory system using intratracheal or intranasal instillation. Ito et al. (2001) evaluated the distribution of purified microcystin-LR after intratracheal instillation of lethal doses in male ICR mice and included a limited description of toxic effects. Microcystin-LR in saline solution was instilled at doses of 50, 75, 100, 150 and 200 µg/kg into 34 mice; three mice were sham-exposed as controls. Mortality was 100% in 12 mice receiving doses of 100 µg/kg and greater. At 75 µg/kg, two of four mice died, while no deaths occurred in 18 mice given 50 µg/kg intratracheally.

The time course of hepatotoxicity was further evaluated in eight mice given an intratracheal dose of 100µg/kg (Ito et al., 2001). One mouse was sacrificed at each of 5, 10, 20, 30, 45, 60, 90 and 120 minutes. Immunostaining for microcystin-LR showed the toxin in the lungs within 5 minutes and in the liver after 60 minutes. Hemorrhage in the liver was observed after 90 minutes and became severe by 120 minutes.

Fitzgeorge et al. (1994) conducted experiments in CBA/BALBc mice with microcystin-LR (commercial product; purity not stated) administered either by intranasal instillation or aerosol inhalation. Few details of study design and findings were given. A single experiment with mice (number unspecified) inhaling a fine aerosol (particle size 3-5 µm) of 50 µg microcystin-LR/L for an unspecified duration of time did not result in any deaths, clinical signs of toxicity or histopathological changes. The nature of the examinations was not reported. The authors estimated the delivered dose of microcystin-LR to be very small (about 0.0005 µg/kg). The LD_{50} for intranasal instillation of microcystin-LR was equal to 250 µg/kg. Liver and kidney weights were increased by 41.6 and 7.5%, respectively, in the animals receiving microcystin-LR intranasally.

Fitzgeorge et al. (1994) further evaluated the relationship between dose and liver weight increase after intranasal instillation of microcystin-LR. At single intranasal doses of 31.3, 62.5, 125, 250 and 500 µg/kg, liver weight increased proportionally (0, 1.5, 24.4, 37.4 and 87%). Seven daily intranasal doses of 31.3 µg/kg, resulted in a liver weight increase of 75%. The authors reported histopathological findings, but failed to specify which findings resulted from single doses and which resulted from the multiple-dose experiment reported in the same publication. Findings included necrosis of respiratory and olfactory epithelium in the nasal mucosa and centrilobular necrosis with hemorrhage in the liver. Early changes in the liver included vacuolar degeneration and necrosis of hepatocytes near the central vein. The adrenal

glands showed effects as well, with vacuolation and necrosis of the inner cortex, as well as congestion of medullary blood vessels. No histopathological changes were observed in the trachea, lungs, esophagus, pancreas, spleen, lymph nodes, kidneys or brain.

Dermal/Ocular Exposure

Commented [IS42]: Stewart *et al* (2006) *BMC Dermatology* 6:5 dosed mouse skin with a *Microcystis* suspension with a high MC-LR content, with no adverse effects seen

No animal studies evaluating the effects in animals of dermal or ocular exposure to purified microcystins were identified. Cyanobacteria bloom samples collected from five different lakes or ponds were tested for allergenic and irritative effects in guinea pigs and rabbits, respectively (Torokne et al., 2001). The microcystin content (presumed to be total LR, RR, and YR) ranged from 0.1-2.21 mg/g. To determine sensitization, guinea pigs were initiated with an intradermal injection followed seven days later by topical application at the injection site, Sensitization was moderate to strong in 30-67% of guinea pigs but did not correlate with microcystin content. All samples produced only negligible to slight skin and eye irritation on rabbits.

7.1.2.2 Short-Term Studies

Oral Exposure

Huang et al. (2011) evaluated the effects of orally administered microcystin-RR on apoptosis in the liver of adult male ICR mice (see also section 7.1.3.5.1). Groups of 5 rats were administered doses of 0, 4.6, 23, 46, 93, or 186 µg/kg body weight of microcystin-RR (commercial product; purity not stated) via gavage for 7 days. After 7 days of exposure, animals were sacrificed and liver slices were prepared for the terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick end-labeling (TUNEL) assay. Homogenized liver tissue was prepared for serine/threonine protein phosphatase 2A (PP2A) activity analysis and PP2A A subunit mRNA expression. Homogenized liver tissue was also analyzed with Western blot for B cell lymphoma/leukemia-2 (Bcl-2), Bcl-2 associated x protein (Bax), p53 expression, C/EBP homologous protein (CHOP), and glucose-related protein 78 (GRP78). There was a dose-dependent increase in the percent of apoptotic cells in the liver (i.e., 10.46, 12.6, 12.7, 30.3, 28.5, and 37.5%, for the 0, 4.6, 23, 46, 93, and 186 µg/kg dose groups, respectively) with statistical significance achieved at doses ≥46 µg/kg. A significant increase in Bax protein expression was found at 46 and 93 µg/kg and in p53 protein expression was found at 93 µg/kg. Bcl-2 was significantly decreased with doses ≥23 µg/kg. The Bax/Bcl-2 ratio was significantly increased with ≥23 µg/kg. No significant changes were found in CHOP protein expression. GRP78 protein expression was significantly increased at 93 µg/kg, but none of the other doses were different from the control (including the high dose). No changes in PP2A activity or alterations in PP2A A subunit mRNA expression were seen.

Heinze (1999) evaluated the effects of microcystin-LR (commercial product; purity not stated) in drinking water on 11-week-old male hybrid rats (F1 generation of female WELS/Fohm x male BDIX). Groups of 10 rats were given doses of 0, 50 or 150 µg/kg body weight for 28 days in drinking water. Water consumption was measured daily and rats were weighed at weekly intervals. Dose estimates provided by the authors were not adjusted to account for incomplete drinking water consumption (3-7% of supplied water was not consumed over the 28-

day period). After 28 days of exposure, rats were sacrificed by exsanguination under ether anesthesia. Organ weights (liver, kidneys, adrenals, thymus and spleen) were recorded and hematology, serum biochemistry and histopathology of liver and kidneys were evaluated.

Hematological evaluation demonstrated an increase in the number of leukocytes in rats in the highest dose group (38% increase). Serum biochemistry showed significantly increased mean levels of ALP and lactate dehydrogenase (LDH) in both treatment groups (84 and 100% increase in LDH, 34 and 33% increase in ALP in low and high doses, respectively) and no changes in mean levels of ALT or AST. A dose-dependent increase in relative liver weights was observed (17 and 26% at the low and high doses, respectively). Table 7-4 shows the mean enzyme levels and relative liver weights.

Table 7- 4. Serum Enzyme Levels and Relative Liver Weights (Mean ± Standard Deviation) in Rats Ingesting Microcystin-LR in Drinking Water

Parameter	Control n=10	50 µg/kg n=10	150 µg/kg n=10
Relative liver weight (g/100 g body weight)	2.75±0.29	3.22±0.34*	3.47±0.49*
Lactate dehydrogenase (microkatals/L)	16.64±4.48	30.64±5.05*	33.58±1.16*
Alkaline phosphatase (microkatals/L)	9.67±2.20	13.00±3.81*	12.86±1.85*

From Heinze, 1999
* $p \leq 0.05$ when compared with control

A dose-dependent increase in absolute liver weight was also reported, but the data were not provided. No statistically significant changes in other organ weights or body weights were observed. The incidence of liver lesions is summarized in Table 7-5. Lesions were spread diffusely throughout the parenchyma and included increased cell volume, increased mitochondria, cell necrosis, activation of Kupffer cells and increased amounts of periodic acid-Schiff (PAS)-positive substances, indicating cell damage. Liver lesions were observed in both treatment groups, but the severity of the damage was increased in the 150 µg/kg dose group. No effects on the kidneys were observed. The low dose (50 mg/kg/day) was a lowest-observed-adverse-effect level (LOAEL) for effects on the liver.

Table 7- 5. Incidence of Liver Lesions in Rats Ingesting Microcystin-LR in Drinking Water for 28 Days

Liver Histopathology	Control (n=10)	50 µg/kg (n=10)	150 µg/kg (n=10)
Degenerative and Necrotic Hepatocytes with Hemorrhage			
Slight	0	4	0
Moderate	0	6	6
Intensive damage	0	0	3
Degenerative and Necrotic Hepatocytes without Hemorrhage			
Slight	0	0	0
Moderate	0	0	1
Intensive damage	0	0	0
PAS-positive Material			
Slight	1	5	0
Moderate	0	5	8
Intensive damage	0	0	2
Activation of Kupffer Cells			
Slight	0	0	0
Moderate	0	10	10
Intensive damage	0	0	0
Lipid Granules and Droplets			
Slight	0	4	0
Moderate	1	2	1
Intensive damage	0	0	0

From Heinze, 1999

Schaeffer et al. (1999) reported the results of a study in which *A. flos-aquae*, a cyanobacterium consumed as a food supplement, was fed to mice in the diet. The authors used recent analysis of the *A. flos-aquae*, which often coexists with *Microcystis* species, to estimate the microcystin content in the material consumed by the mice. Analysis of the *A. flos-aquae* samples used in the feeding study showed an average concentration of 20±5 µg MC-LR per gram of *A. flos-aquae*. The authors estimated the daily exposure of microcystin-LR in the exposed mice to range from 43.3 µg/kg-day to 333.3 µg/kg-day. No clinical signs of toxicity were reported, and no effects on mortality, body weight, organ weights or histology were observed in the treated mice. In addition, no effects on reproductive parameters were reported in five treated mice from the highest dose group allowed to breed. The 333.3 µg/kg-day dose was a no-observed-adverse-effect level (NOAEL) under the conditions of the study.

Inhalation Exposure

Benson et al. (2005) exposed groups of six male BALB/c mice to monodispersed submicron aerosols of MC-LR via nose-only inhalation for 30, 60 or 120 minutes each day for seven consecutive days. The concentration of MC-LR was 260-265 µg/m^3 and doses deposited in the respiratory tract were estimated to be 3, 6 and 12.5 µg/kg body weight/day. Control mice were exposed to the aerosolized vehicle (20% ethanol in water). Clinical signs were recorded daily. Sacrifice by injection of Euthasol occurred the day after the last exposure. Blood was

collected by cardiac puncture and serum was subjected to clinical chemistry analysis (blood urea nitrogen [BUN], creatinine, total bilirubin, ALP, AST, ALT, total protein, albumin and globulin). Organ weight (adrenals, lung, liver, kidney, spleen and thymus) was recorded and histopathological examination of the liver, respiratory tract tissues, adrenals, kidney, spleen, thymus, gastrointestinal tract and testes was conducted.

No clinical signs or effects on body weight or organ weights were observed following exposure to microcystin-LR aerosol. Histopathological examination revealed treatment-related lesions in the nasal cavity only. Lesions were not observed in the liver, other organs or in other parts of the respiratory tract. As indicated in Table 7-6, the incidence and severity of nasal lesions increased with length of the daily exposure period. The lesions consisted primarily of necrosis or inflammation of respiratory epithelial cells and degeneration, necrosis and atrophy of olfactory epithelial cells. Necrotic lesions of olfactory epithelial cells were generally larger patches, while few cells were involved in respiratory epithelial cell necrosis.

Table 7- 6. Incidence and Severity of Nasal Cavity Lesions in Mice Inhaling Microcystin-LR Aerosol for 7 Days

Lesion	Severity	Control	Daily Exposure Period (minutes)		
			30	60	120
Respiratory Epithelial Necrosis					
Turbinate 1	Minimal	0/6	1/6	0/6	0/6
	Mild	0/6	0/6	6/6	0/6
	Moderate	0/6	0/6	0/6	2/6
Turbinate 2	Mild	0/6	0/6	6/6	3/6
	Moderate	0/6	0/6	0/6	3/6
Respiratory Epithelial Inflammation					
Turbinate 1	Mild	0/6	1/6	0/6	1/6
Turbinate 2	Mild	0/6	1/6	0/6	0/6
Olfactory Epithelial Degeneration, Necrosis and Atrophy					
Turbinate 1	Mild	0/6	0/6	0/6	4/6
	Moderate	0/6	0/6	0/6	1/6
Turbinate 2	Mild	0/6	0/6	6/6	0/6
	Moderate	0/6	0/6	0/6	6/6
Turbinate 3	Mild	0/6	0/6	6/6	0/6
	Moderate	0/6	0/6	0/6	4/6
	Marked	0/6	0/6	0/6	2/6

From Benson et al., 2005

7.1.2.3 Subchronic Studies

Oral Exposure

Fawell et al. (1999a) reported the results of a subchronic toxicity study of MC-LR given via gavage to Cr1:CD-1(ICR)BR (VAF plus) mice (age not specified). MC-LR was obtained commercially (purity not stated) and administered in distilled water. The concentration in the dosing solution was verified by HPLC with UV detection. Daily oral gavage doses of 0, 40, 200 or 1000 µg/kg body weight were given to groups of 15 male and 15 female mice for 13 weeks. Daily clinical observations were made, body weight and food consumption were recorded weekly, and eye examinations were conducted prior to and at the conclusion of treatment. Hematology and serum biochemistry were evaluated for seven mice of each treatment group during the final week of treatment. Upon sacrifice after 13 weeks, gross examination of organs and microscopic evaluation of tissues were performed. All tissues were examined in the control and high dose animals, while only lungs, liver and kidney were examined in the other treated animals.

At 1000 µg/kg, one female was found dead during week 1 and one male was killed moribund during week 13; a cause of death was not given and both animals appeared to be included in histopathology analyses. No treatment-related clinical signs of toxicity were observed throughout the study. Mean body weight gain was decreased approximately 15% in all treated male groups. Mean terminal body weights differed from controls by about 7% in these groups. No dose-related trends were evident for body weight gain or body weight in males. The only body weight change observed in females was an increase in body weight gain in the 200 µg/kg-day group. Hematological evaluation showed slight (10-12%) increases in mean hemoglobin concentration, red blood cell count and packed cell volume among females receiving 1000 µg/kg body weight. ALP, ALT and AST levels were significantly elevated (2- to 6-fold higher) in the high-dose males, and ALP and ALT were likewise elevated (2- and 6-fold higher, respectively) in high dose females. ALT and AST were also elevated (2-fold) in the mid-dose males. GGT was slightly decreased in some treatment groups. Serum albumin and protein were reduced (13%) in males of the mid- and high-dose groups. Table 7-7 shows the clinical chemistry results.

Histopathological changes in the liver were reported in the males and females of the mid- and high-dose groups, with a dose-related increase in incidence and intensity. The liver lesions were multifocal inflammation with deposits of hemosiderin and hepatocyte degeneration throughout the liver lobule. Table 7-8 summarizes the incidence of liver histopathological changes. Sex-related differences in liver pathology were not apparent. No lesions were found in other tissues. The NOAEL was 40µg/kg/day and the LAOEL 200 µg/kg/day for liver histopathology and elevated serum levels of ALT and AST in males.

Table 7- 7. Blood Chemistry Results (Mean ± Standard Deviation) for Mice Treated with Microcystin-LR for 13 Weeks

MC-LR Dose (µg/kg-day)	Alkaline Phosphatase (ALP) (U/L)	Alanine Aminotransferase (ALT) (U/L)	Aspartate Aminotransferase (AST) (U/L)	Gamma Glutamyl Transaminase (GGT) (U/L)	Total Protein (g %)	Albumin (g %)
Male						
Control	91±22.2	27±8.0	68±27.7	6±1.0	5.5±0.32	3.2±0.19
40	95±29.2	37±17.2	64±12.2	4±0.7	5.1±0.26	3.0±0.13
200	94±32.3	59[a]±28.0	121[b] ±43.7	3[c]±0.4	4.8[b]±0.29	2.8[c]±0.13
1000	232[b]±103.2	159[c]±75.0	121[b] ±26.3	4±0.4	4.8[c]±0.21	2.8[c]±0.11
Female						
Control	167±24.6	32±11.3	101±38.3	4±1.0	5.1±0.30	3.1±0.14
40	187±76.2	25±7.8	74±13.2	3±0.5	5.2±0.28	3.2±0.16
200	156±33.4	27±9.4	74±22.1	3±0.0	5.3±0.31	3.4[a]±0.14
1000	339[b]±123.7	220[b]±149.1	144±71.7	3±0.4	5.1±0.22	3.1±0.18

From Fawell et al., 1999a

Significantly different from controls at: [a] p<0.05; [b] p<0.01; [c] p<0.001.

Table 7- 8. Incidence of Liver Histopathology in Mice Treated with Microcystin-LR for 13 Weeks

Liver Histopathology	Control	40 µg/kg-day	200 µg/kg-day	1000 µg/kg-day
Male	n=15	n=15	n=15	n=15
Acute inflammation	0	1	0	0
Chronic inflammation	1	2	4	15
Congestion	3	0	0	1
Hepatocyte vacuolation	5	5	6	3
Hemosiderin deposits	0	0	0	15
Hepatocyte degeneration	0	0	1	14
Female	n=15	n=15	n=15	n=15
Autolysis	0	0	0	1
Chronic inflammation	5	8	8	14
Congestion	0	0	0	1
Hepatocyte vacuolation	5	5	11	8
Hemosiderin deposits	0	0	1	14
Hepatocyte degeneration	0	0	1	9

From Fawell et al., 1999a

Falconer et al. (1994) administered dried bloom materials in the drinking water of pigs (n= 5/group) for 44 days. The extract contained at least seven microcystin variants with the major peak tentatively identified as MC-YR; no peak could specifically be identified as MC-LR. Animals were administered 0, 80, 227, or 374 mg of dried algae/kg body weight-day added to the drinking water. Pigs in the highest dose group had reduced body weight likely due to reduced food and/or water consumption at this dose. Plasma samples collected over 56 days showed dose- and time-dependent increases in GGT, ALP and total bilirubin, as well as a

decrease in plasma albumin. Dose-related changes in the incidence and severity of histopathological changes of the liver were also observed, including cytoplasmic degeneration, hepatic cord disruption, single cell necrosis, periacinar degeneration, congestion, and Kupffer cell proliferation. The study does not identify a NOAEL or LOAEL for microcystin because the exposure was to the dried algae.

Inhalation Exposure

No data from subchronic inhalation exposure of animals were found.

7.1.2.4 Neurotoxicity

No reports of neurotoxicity by the oral or inhalation routes of exposure were identified. Two of the studies that follow used intrahippocampal injection, a third was an *in vitro* study with frog muscle, and a fourth was an in vitro study using isolated neurons.

Maidana et al. (2006) reported that long-term memory retrieval, as assessed by step-down inhibitory avoidance task, was impaired in rats receiving an intrahippocampal injection of 0.01 or 20 µg/L of a microcystin extract from *Microcystis* strain RST 9501. Exposure to 0.01µg/L also impaired spatial learning in the radial arm maze, but exposure at the higher concentration did not. The authors indicated that the primary microcystin produced by this strain is [D-Leu^1] MC-LR, a variant of MC-LR. Oxidative damage, as measured by lipid peroxides and DNA damage, was increased in tissue homogenates of the hippocampus from treated animals.

Commented [IS43]: So no dose-response findings here?

Li et al. (2012) also reported impaired memory function, assessed by Morris water maze, in male rats receiving intrahippocampal injection of 1 or 10 µg/L of MC-LR (≥98% pure). Both concentrations of MC-LR caused increased latency to find the platform. Histology of the brain revealed neuronal damage in the CA1 region of the hippocampus at 10µg/L only. A significant decrease in the total number of cells and the density of cells, but not in the cell volume, was seen in the CA1 region of high-dose animals. Malondialdehyde (MDA) levels and catalase activity in the hippocampal CA1 region were increased at both concentrations, but superoxide dismutase (SOD) and glutathione peroxidase activity were only significantly increased at 10 µg/L.

Foxall and Sasner (1981) conducted limited *in vitro* studies on the neurological effects of a crude extract from a bloom of *M. aeruginosa*. Few details on experiment design were reported; frog and mouse heart, frog sartorius muscle, frog sciatic nerve and mouse ileum were used in the experiments. The authors reported that the extract had no effect on electrical or mechanical events in the isolated from muscle.

Feurstein et al. (2011) examined the effects of microcystin-LR on isolated murine cerebellar granule neurons. Cell viability was significantly decreased at 5 µM MC-LR, but apoptosis was not induced by concentrations up to 5 µM. Capase-3/7 activity was also not increased with concentrations up to 5 µM. Slight impairment of the neurite network was observed in the cells incubated at ≥1 µM MC-LR for 48 hours, and a significant dose-related decrease in neurite length was observed at concentrations ranging from 1-10 µM. This was

accompanied by serine/threonine-specific PP inhibition and sustained hyperphoshorylation of Tau.

7.1.2.5 Developmental/Reproductive Toxicity

Reproductive Effects

Oral

Sperm quality and testicular function were assessed in male SPF mice (0.015-0.025 kg at purchase) administered MC-LR (commercial produce; purity not stated) in the drinking water at concentrations of 0, 1, 3.2, or 10 µg/L for 3 or 6 months (Chen et al., 2011). Although body weight and amount of water consumed were measured, these data were not presented and doses to the animals were not calculated. Based on the subchronic reference drinking water value of 0.0078 L/day and body weight of 0.0316 kg for the male B6C3F1 mouse (U.S. EPA, 1988), doses to the animals were estimated as 0, 0.25, 0.79, and 2.5 µg/kg. Subchronic reference values were chosen to more accurately reflect status of the animals after 6 months of treatment; based on growth curves for the B6C3F1 mouse and initial body weights of the SPF mice, the B6C3F1 strain was considered reasonably similar to the strain used in this study.

Commented [IS44]: I can't see that the mouse strain was reported in this study. SPF in this context merely abbreviates "specific pathogen-free" and might confuse the reader who might otherwise think SPF is a mouse strain.

No clinical signs of toxicity were observed and body weight, testes weight, and water consumption were not affected by treatment. Results of sperm and hormone analyses are shown in Table 7-9. No significant changes in any parameter were noted at 1 µg/L. At 3.2 and 10 µg/L, sperm counts were significantly decreased and sperm motility was reduced at 3 and 6 months with severity increasing with the longer duration of exposure. Animals in the mid- and high-dose groups had a trend towards lower serum testosterone and higher luteinizing hormone and follicle stimulating hormone after 3 months which reached statistical significance by 6 months. Histopathological evaluation of the testes showed a slightly loosened appearance of the organization of the epithelium in the seminiferous tubules at 10 µg/L after 3 months. After 6 months, slight testicular atrophy associated with sparse appearance of the seminiferous tubules was found at 3.2 and 10 µg/L with dose-related increased severity. The animals given 10 µg/L also showed loss and derangement of spermatogenic cells, enlargement of the lumen of the seminiferous tubules, thinning of the spermatogenic epithelium, as well as depopulation of Leydig cells, Sertoli cells, and mature sperm. The number of apoptotic cells in the testes was increased at 10 µg/L after 3 months and at 3.2 and 10 µg/L after 6 months. The NOAEL was 0.27 µg/kg/day and the LOAEL was 0.86 mg/kg/day.

Table 7- 9. Serum hormone levels and sperm analyses from mice given Microcystin-LR in the drinking water for 3 or 6 months

Endpoint	0 µg/kg/day	0.25 µg/kg/day	0.79 µg/kg/day	2.5 µg/kg/day
3 months				
Testosterone (ng/mL)	2.23 ± 1.15	2.77 ± 0.93	2.34 ± 1.11	1.07 ± 0.27
LH (mIU/mL)	7.03 ± 0.41	7.28 ± 0.66	8.05 ± 0.37	7.71 ± 0.27
FSH (mIU/mL)	3.05 ± 0.14	3.12 ± 0.36	3.37 ± 0.32	3.49 ± 0.47
Sperm count ($\times 10^6$/mL)	27.0 ± 1.5	23.5 ± 0.8	17.8 ± 1.5**	13.3 ± 1.3**
Sperm motility (%)	71.7 ± 3.3	57.6 ± 5.5	54.0 ± 6.4*	34.6 ± 3.3**
Abnormal sperm (%)	5.9 ± 1.0	5.9 ± 1.0	6.1 ± 0.9	6.5 ± 1.0
6 months				
Testosterone (ng/mL)	3.33 ± 0.98	2.03 ± 0.73	1.08 ± 0.17**	0.89 ± 0.29**
LH (mIU/mL)	4.89 ± 0.25	4.84 ± 0.25	5.88 ± 0.25*	5.66 ± 0.17**
FSH (mIU/mL)	2.36 ± 0.35	2.59 ± 0.37	3.16 ± 0.32	4.27 ± 0.52**
Sperm count ($\times 10^6$/mL)	21.5 ± 0.7	19.7 ± 0.9	13.6 ± 1.1**	6.6 ± 0.9**
Sperm motility (%)	60.6 ± 5.1	46.8 ± 6.7	23.1 ± 3.2**	17.4 ± 5.0**
Abnormal sperm (%)	6.5 ± 1.0	9.0 ± 1.0	13.8 ± 1.8**	14.5 ± 1.1**

From Chen et al., 2011 Data are mean±S.E.; n = 10.
Significantly different from control: *p<0.05; **p<0.01.

Kirpenko et al. (1981) collected a natural population of *M. aeruginosa* from a reservoir during the summer months. Male and female white rats (total of 120 rats) were intubated with 5×10^{-4} or 5×10^{-7} mg/kg of toxin extracts (it was not specified what was in the extract) or 10 mg/kg of *M. aeruginosa* biomass for three months (dosing procedure not specified). Estrous cycle and microscopic studies of the genital appendages and testes in males, as well as histopathology of the ovaries and testes were conducted. Changes in the estrous cycle were observed with 5×10^{-4} mg/kg of toxin extract or 10 mg/kg of biomass. Growth and maturation of the oocytes was also affected. Degeneration of oocytes in Graafian vesicles, decreased follicle dimensions, and increased number of involuted corpora lutea were observed with 5×10^{-4} mg/kg of the toxin extract. In males, there was a decrease in spermatozoid motility, living spermatozoids (increased dead), spermatogonia quality, and spermatid quality with a dose of 5×10^{-4} mg/kg of toxin extract. Histological evaluation revealed greater tubule deformation and "epithelium shelled out" (not defined) from basal membranes. Effects on Sertoli cells and spermatogonia were also noted.

Falconer et al. (1988) conducted a limited study of reproductive effects using an extract from an *M. aeruginosa* bloom sample. Eight female mice that had been given 1/4th dilution of the extract as drinking water (estimated to contain 14 µg/mL of unspecified microcystin toxin)

since weaning were mated with similarly treated males. No difference in number of litters, pups per litter, sex ratio, or litter weight were observed. Reduced brain size was reported to occur in seven of 73 pups from treated parents and in none of 67 pups from controls. The litter distribution of the affected pups was not reported by the authors. One of the small brains was examined histologically, revealing extensive damage to the hippocampus.

Other Routes

Cellular damage was observed in the testes of male mice administered a single i.p. dose of 55-110 µg MC-LR/kg prepared from a crude extract of a lyophilized cyanobacterial bloom (Li et al., 2011b). The effects of a single i.p. injection of microcystin extracts from a surface bloom containing 167.7 µg MC-RR/mL and 47.0 µg MC-LR/mL or 80.5 MC-LR equivalents/mL was found to have an effect on male rabbit testes. Lesions, including a variety of histological changes to both spermatogonia and Sertoli cells, were seen in animals treated with 12.5 µg MC-LR equivalents/kg; recovery occurred by 48 hours with the tissue resembling the control (Liu et al., 2010).

Male reproductive effects have also been consistently observed after short-term parenteral exposures. These studies are described below.

In a study by Chen et al. (2013), male rats (10 per group) were i.p. injected with MC-LR (purity ≥ 98%) for 50 days at doses of 1 or 10 µg/kg/day; a control group (n =10) was injected with the same volume of 0.9% saline solution. Animals were sacrificed twelve hours following the final injection and the testes removed. The relative testes weight was significantly decreased ($p < 0.01$) at 10µg/kg/day, however, body weight and absolute organ weight data were not given. Light microscopic observations indicated that the space between the seminiferous tubules and lumen size increased with increasing dose; blockages in the seminiferous tubules were also reported at 10 µg/kg/day. Ultrastructural observations in spermatogonia showed some abnormal histopathological characteristics, including cytoplasmic shrinkage, cell membrane blebbing, swollen mitochondria and deformed nucleus; these changes became more pronounced with increasing dose. Using qPCR methods, the transcriptional levels of select cytoskeletal and mitochondrial genes were determined. MC-LR exposure affected the homeostasis of the expression of cytoskeletal genes, causing possible dysfunction of cytoskeleton assembly. Transcription of β-actin, β-tubulin, and stathmin were significantly decreased while ezrin and moesin were increased. In both MC-LR treated groups, all 8 mitochondrial genes related to oxidative phosphorylation (OXPHOS) were significantly increased. The levels of reactive oxygen species (ROS) were significantly increased ($p < 0.01$) at 10 µg/kg/day as was mitochondrial swelling and DNA damage. Changes in testicular hormone levels included increased FSH levels at 10 µg/kg/day, significantly increased LH levels in both treated groups ($p < 0.05$ or 0.01), and decreased testosterone levels in both dose groups ($p < 0.01$) compared to those of the controls. The authors concluded that this study provides evidence that both cytoskeleton structural disruption and mitochondrial dysfunction interact through inducing of reactive oxygen species and oxidative phosphorylation resulting in testis impairment following exposure to MC-LR.

Ding et al. (2006) examined the effects of microcystin from an extract of *Microcystis* (99.5% MC-LR, 66.476 µg/mL, and 0.5% MC-YR, 0.361 µg/mL) on the reproductive system of male mice administered 0, 3.33, or 6.67 µg microcystin/kg i.p. daily for 14 days. The high-dose group had a decrease in body weight during the course of the study. Both treatments had a significant decrease in body weight gain. There was a dose-dependent decrease in absolute testes weight, but significant increase in relative testes weight in the high-dose group. There was a significant decrease in absolute and relative epididymis weight in the high-dose group. There was a dose-dependent decrease in sperm viability and the proportion of sperm with rapid progressive motility. The high-dose group also had an increase in the percent immobile sperm. There was no increase in the percent of abnormal sperm. Histologically both treatment groups had atrophy of the seminiferous tubules with increased spacing between the seminiferous tubule cells. The effect increased with increasing dose. The high-dose group also exhibited deformation of androgonial and sperm mother cells, and decreased number of interstitial cells, Sertoli cells, and mature sperm in the seminiferous tubules.

Li et al. (2008) also observed male reproductive effects in male Sprague-Dawley rats administered 0, 5, 10, or 15 µg MC-LR/kg-day for 28 days. Body weight gain was decreased in all treatment groups. Absolute and relative testes weights were decreased in the high-dose group. High-dose animals had decreased epididymal sperm concentrations. Sperm motility was significantly decreased and the percent of abnormal sperm increased in all dose groups. Serum testosterone levels were significantly decreased in both 10 and 15 µg/kg-day dose groups. Both FSH and LH were significantly increased at 5 µg/kg-day, but significantly decreased at 15 µg/kg-day. Histopathological changes in the testes occurred in all MC-LR treated groups, but were more pronounced in the high-dose group, including atrophied and obstructed seminiferous tubules.

Developmental Effects

Fawell et al. (1999a) reported the results of a developmental toxicity study of MC-LR (commercial product; purity not stated) given via gavage to Cr1:CD-1(ICR)BR (VAF plus) mice. MC-LR (0, 200, 600 or 2,000 µg/kg/day) was administered to groups of 26 mice on days 6-15 of pregnancy. The mice were sacrificed on day 18 and necropsied. Weight and sex of the fetuses were recorded, and external, visceral and skeletal examinations performed. Seven of 26 dams receiving 2,000µg/kg/day died and 2 others were sacrificed moribund. Altered liver appearance was noted during gross examination of these animals. Surviving dams in this group did not display any clinical signs of toxicity or differences in body weight or food consumption. The authors reported that fetal body weight was significantly lower than controls and there was delayed skeletal ossification at the highest dose; however, the data were not presented in the publication. No effects on resorptions or litter size were observed, nor were there increases in external, visceral or skeletal abnormalities in fetuses of any treatment group. The apparent NOAEL is 600 µg/kg/day with a LOAEL of 2,000 µg/kg/day for low fetal body weight and decreased skeletal ossification.

Groups of 6-8 timed-pregnant CD-1 mice were administered MC-LR (commercial product; 95% purity) in sterile saline by i.p. injection at doses of 0, 32, 64, or 128 µg/kg. Animals were treated on gestation days 7-8, 9-10, or 11-12 followed by sacrifice on day 17.

Fetuses were examined for gross and skeletal malformations (Chernoff et al., 2005). Maternal weight change, pregnancy rate, litter size, fetal deaths, and fetal body weight were similar between control and treated groups. No treatment-related malformations were found on fetal examination.

In another part of the Chernoff et al. (2005) study, pregnant CD-1 mice were administered MC-LR (commercial product; 95% purity) in sterile saline by i.p. injection at doses of 0, 32, 64, 96, or 128 µg/kg. Animals were treated on gestation days 7-8, 9-10, or 11-12, and allowed to give birth. The growth and viability of pups was monitored for 5 days. A different lot of MC-LR from the same supplier was used in this part of the study and was much more toxic than the lot used in the developmental toxicity study. Maternal deaths were observed at all doses independent of days of dosing. In the control and treated groups, 0/25, 3/27, 19/35, 33/34, and 33/34 animals died, respectively. For surviving dams, numbers of pups born, and offspring survival and body weight through postnatal day 5 were not affected by treatment.

7.1.2.6 Chronic Toxicity

Ueno et al. (1999) evaluated the toxicity of microcystin-LR in mice chronically exposed via drinking water. Two hundred 6-week-old female BALB/c mice were randomly assigned to receive either no treatment or drinking water (*ad libitum*) containing 20 µg/L MC-LR for 7 days/week for up to 18 months. The MC-LR had been isolated from lyophilized algal bloom materials from Lake Suwa in Nagano, Japan and had been characterized as 95% pure by HPLC. Twenty animals from each group were sacrificed at 3, 6 and 12 months, while the remaining 40 animals were retained for chronic toxicity evaluation and sacrificed at 18 months.

Weekly estimates of food and water consumption and daily observations for clinical signs of toxicity, morbidity and mortality were recorded. Body weights were recorded weekly for the first 2 months, biweekly up until the first year and monthly until sacrifice. At 3, 6, 12 and 18 months, blood was obtained from 20 animals from each group. Samples from 10 animals per group were used for hematological evaluation, and samples from 10 additional animals were used for serum biochemistry evaluation. At each scheduled sacrifice time, complete necropsy of 10 animals per group was conducted. Animals from the chronic toxicity group were necropsied when moribund or found dead prior to scheduled sacrifice or upon sacrifice at 18 months. Relative and absolute organ weights (liver, kidneys, spleen, thymus, adrenal, ovaries, brain, heart and uterus) were recorded for 9-10 animals per group at each scheduled sacrifice, and histopathological evaluation of these and numerous other organs was conducted. Finally, three to five animals per group were subjected to immunohistochemistry of the liver upon sacrifice to determine the distribution of MC-LR in the liver.

Based on weekly estimates of water consumption, the cumulative intake of MC-LR over 18 months was calculated to be 35.5µg/mouse. No clinical signs of toxicity were observed, and survival in the control and chronic treatment groups was similar. No statistically significant differences in body weight, food consumption, water consumption or hematology were observed; however, hematology data from the 3-month sacrifice were lost due to sampling errors. Treated mice were reported to have a statistically significant decrease in serum ALP at month 12 (13%) and a significant increase in cholesterol at month 18 (22%). Neither effect was considered by the

authors to be toxicologically significant in the absence of other treatment-related effects; however, the increase in cholesterol could be related to interference of MC-LR with bile acid transports in the liver.

A decrease in heart weight among treated mice sacrificed at month 12 was not considered treatment-related in the absence of histopathological changes. Sporadic changes in absolute and relative thymus weight in treated mice were observed, but histological and morphometric evaluation of the thymus revealed no abnormalities attributable to exposure. In contrast to other studies, the authors observed no difference in the incidence of liver histopathology between treated and control mice. Immunohistochemistry of the liver revealed no accumulation of MC-LR.

Ito et al. (1997b) evaluated the carcinogenicity and liver toxicity of chronic gavage doses of MC-LR. A water bloom from Lake Suwa, Japan served as the source of the MC-LR, which was isolated and dissolved in ethanol and saline for dosing. The purity of the isolated MC-LR was not specified. Twenty-two ICR mice (13 weeks old) were given either 80 or 100 gavage doses of 80 µg MC-LR/kg/day over the course of 28 weeks. Ten mice were sacrificed after 80 treatments, five were sacrificed after 100 treatments and seven were withdrawn from treatment after 100 doses and sacrificed 2 months later. Three mice served as untreated control. Although the authors did not specify the nature of the postmortem examinations, it appears that the liver was the only organ examined. No change in mean liver weight was observed in the MC-LR-treated animals compared with controls. The authors reported "light" injuries to hepatocytes in the vicinity of the central vein in 8 of 15 mice sacrificed immediately after treatment, and in 5 of 7 mice that were withdrawn from treatment for 2 months after exposure. No fibrous changes or neoplastic nodules were observed. Analysis for MC-LR and its metabolites by immunohistochemistry failed to detect either the parent compound or any metabolites in the livers of mice sacrificed immediately after treatment.

Falconer et al. (1988) conducted a chronic exposure experiment (up to 1 year) using an extract of a *M. aeruginosa* water bloom in Swiss Albino mice. A concentration-dependent increase in mortality, reduced body weight and a concentration-dependent increase in serum alanine aminotransferase levels were observed among groups of mice receiving serial dilutions of the extract as their drinking water for a year. There was some evidence that bronchopneumonia incidence was related to concentration of extract. No significant differences in liver histopathology were observed when compared to the control, although the observed liver changes (neutrophil infiltration, hepatocyte necrosis) were slightly more prevalent in treated animals. The data showed some indication of sex differences in susceptibility; male mice showed effects (including mortality and serum enzyme level increases) at lower concentrations than females.

Thiel (1994) briefly reported the results of a chronic toxicity study of MC-LA in vervet monkeys. The report is a brief summary published in the proceedings of an international workshop; a published version of this study was not located. According to the summary, three monkeys were given increasing intragastric doses of MC-LA for 47 weeks, while three other monkeys served as controls. Doses increased from 20 µg/kg/day at the commencement of the study to 80 µg/kg/day at study termination. The rate of dosage increase was not reported.

Monthly measures of body weight and clinical signs (respiration, pulse, temperature) showed no effect of treatment. Blood was withdrawn monthly; hematological parameters examined were hematocrit, bilirubin, hemoglobin, erythrocyte and leukocyte count and platelet count. No statistically significant changes in hematological parameters were observed. No changes were observed in serum biochemistry analyses (albumin, globulins and electrolytes, as well as serum AST, LDH, ALP, ALT and GGT). Histopathological examination of the liver and other organs (not specified) did not show any differences in treated monkeys when compared with controls.

Zhang et al. (2010) administered MC-LR (commercial product; ≥95% purity) to 8-week old male C57bl/6 mice (10/treatment group) via drinking water at concentrations of 0, 1, 40, or 80 µg/L for 180 days. The doses were reported as 0, 0.2, 8.0, and 16 µg/kg/day, but the method of calculation was not given. Body weight was measured at study initiation and at study termination. At sacrifice, livers were removed and processed for routine (hematoxylin-eosin) or immunohistochemical staining to measure metalloproteinase (MMP[3]) expression. Other liver portions were homogenized for measurement of MMP protein and mRNA levels.

A significant ($p < 0.01$) decrease in body weight, accompanied by an increase in relative liver weight, was reported at 8.0 and 16.0 µg/kg/day (data were not given). Histopathology revealed infiltrating lymphocytes and fatty degeneration in the liver of mice treated with 8.0 and 16.0 µg/kg/day, but incidence and severity data were not provided. There was a significant increase in the area stained positive for MMP9 in all treatment groups and for MMP2 at 8.0 and 16.0 µg/kg/day. The concentrations of MMP9 protein also were increased at all doses, but the MMP2 protein concentration was only significantly increased in the high-dose group. Messanger RNA expression for both MMPs was significantly increased in the mid- and high-dose groups. MC-LR also increased the phosphorylation extracellular signal-regulated protein kinase (ERK) 1/2 and p38 (members of the mammalian of the mitogen-activated protein kinase (MAPK) family).

In a subsequent study, Zhang et al. (2012) administered MC-LR (commercial product; ≥95% purity) to 8-week old male C57bl/6 mice (10/treatment group) via drinking water at concentrations of 0, 1, 40, or 80 µg/L for 270 days (0, 0.2, 8.0, and 16 µg/kg/day). Body weight was measured at study initiation and at study termination. At sacrifice, livers were removed and processed for routine or immunohistochemical staining to measure metalloproteinase (MMP) expression. Other liver portions from five randomly selected mice were homogenized for MMP protein and mRNA levels. No differences in water consumption were found between the groups. Body weight results were not reported. Histopathology revealed infiltrating lymphocytes and fatty degeneration in the livers of mice (doses not specified). MMP expression and protein levels for both MMP2 and MMP9 were significantly increased in all dose groups. MMP mRNA levels were increased in all dose groups for MMP2 and in the mid- and high-dose groups for MMP-9. The changes in MMP expression and protein levels are not considered adverse.

[3] Matrix metalloproteinases are a family of zinc requiring matrix-degrading enzymes, which include the collagenases, gelatinases, and the stromelysins, all of which have been implicated in invasive cell behavior (Brooks et al. 1996. http://www.sciencedirect.com/science/article/pii/S0092867400812350).

7.1.2.7 Carcinogenicity

Oral Exposure

No oral cancer bioassay was found in which animals were administered microcystins or an extract.

Other Routes of Exposure

IARC (2010) summarized studies in rats and mice in which MC-LR or microcystins, administered by i.p. injection, promoted preneoplastic lesions in the liver. These studies are briefly described below.

A group of 13 male ICR mice, 5 weeks of age, received 100 i.p. injections of 20 µg/kg bw of microcystin-LR (five times a week) over 20 weeks and were killed after the end of the treatment (five mice) or after a 2-month withdrawal period (eight mice). Three non-treated mice were used as controls. Neoplastic nodules were found in the liver of all 13 treated mice. Hepatocytes from the most common type of nodule were described as having weakly staining cytoplasm and small nucleus (Ito et al., 1997b).

Groups of 10-19 male Fischer 344 rats, 7 weeks of age, were given a single i.p. injection of 0 or 200 mg/kg of *N*-nitrosodiethylamine (NDEA) in saline followed 2 weeks later by i.p. injections of 0, 1 or 10 µg MC-LR/kg twice a week for 6 weeks and partial hepatectomy at the end of week 3. A subset continued to receive injections of 10, 25, or 50 µg MC-LR/kg twice a week for 5 weeks. Phenobarbital (0.05%) was used as a positive control. The tumor-promoting activity was estimated by induction of glutathione *S*-transferase placental form-positive (GST-P) foci in rat liver. Rats from the groups treated with NDEA plus 10 µg MC-LR/kg both with and without continued treatment after partial hepatectomy had an increased incidence of GST-P foci per liver compared with NDEA-treated rats (Nishiwaki-Matsushima et al., 1992). GST-P foci are considered to be biomarkers for early stage development of potential liver tumors.

Groups of male Fischer 344 rats (initial number unspecified), 7 weeks of age, received a single i.p. injection of 0 or 200 mg NDEA/kg in saline followed 2 weeks later by 20 i.p. injections of 0 or 25 µg MC-LR/kg. Animals treated with NDEA plus MC-LR had significant increases in the number, area, and volume of GST-P-positive foci per liver compared to NDEA-treated rats (Ohta et al., 1994).

Groups of male Fischer 344 rats, 6 weeks of age, received an i.p. injection of 0, 200 mg NDEA/kg, or 0.5 mg aflatoxin B_1/kg two weeks before i.p. injections of 0, 1 or 10 µg MC-LR/kg twice a week for 6 weeks. Other groups were also treated with aflatoxin B_1 plus NDEA before MC-LR treatment. A subset of each treatment scenario was given partial hepatectomy one week after initiation of MC-LR administration. The number and area of GST-P positive foci per liver were increased in rats given NDEA plus aflatoxin B_1; NDEA, aflatoxin B_1, plus MC-LR; and aflatoxin B_1 plus MC-LR. A clear dose-response relationship for the number of foci to the dose of MC-LR was not apparent (Sekijima et al., 1999). Control groups for NDEA and aflatoxin B_1 alone were not included.

7.1.3 Other Key Data

7.1.3.1 Mutagenicity and Genotoxicity

The available data on mutagenicity and genotoxicity of cyanobacterial toxins, including microcystins, has been recently reviewed (Žegura et al., 2011). These authors concluded that current evidence indicates that the microcystins are not bacterial mutagens and that discrepancies in results from cyanobacterial extracts are likely due to differences in source of the cyanobacteria and composition of the complex extract mixtures. Both *in vitro* and *in vivo* genotoxicity studies have shown positive results with DNA damage induced by formation of reactive oxygen species as well as inhibition of repair pathways. These studies are summarized below and listed in Tables 7-10, 7-11, and 7-12.

Mutagenicity

Pure MC-LR did not induce mutations in the Ames assay (strains TA97, TA98, TA100, and TA102) either with or without metabolic activation, while extracts from *Microcystis* exhibited mutagenic activity in the absence of activation which was decreased slightly in TA98 with activation (Ding et al., 1999; Huang et al., 2007). A crude toxin extracted from *M. aeruginosa* did not induce mutations in the Ames assay (strains TA98 and TA100) with and without activation (Grabow et al., 1982). Wu et al. (2006) tested the mutagenicity of MC-LR extracted from a *M. aeruginosa* bloom using three assays (ara test in *E. coli* UC1121, Ames test in *S. typhimurium* strains TA98 and TA100, and SOS/umu test in *S. typhimurium* TA1535/pSK1002). All tests were negative both with and without metabolic activation. Repavich et al. (1990) reported that Ames assays (using strains TA98, TA100 and TA102) of a purified hepatotoxin (supplied by Wright State University and presumed to be microcystin) were negative with and without metabolic activation, as were *Bacillus subtilis* multigene sporulation assays.

In contrast, Suzuki et al. (1998) observed increased ouabain resistance mutation frequency in human embryo fibroblast cells treated with MC-LR (purity not specified). Similarly, Zhan et al. (2004) reported a 5-fold increase over control in the frequency of thymidine kinase mutations when human lymphoblastoid TK6 cells were treated with commercially-obtained MC-LR. More slow-growing mutants were observed than fast-growing mutants, suggesting that the mutation damage was larger than the TK locus, and that MC-LR induced large deletions, recombinations or rearrangements.

The differences in mutagenicity response between bacteria and human cell lines may be related to differences in the cell uptake of MC-LR. For example, the failure of MC-LR to induce mutations in bacterial cells may be related to poor uptake. Zhan et al. (2004) reported that MC-LR is not taken up by many cell types, including bacteria; however, the authors did not provide references to support this assertion. While hepatocytes take up MC-LR at a significant rate, other cell types show limited or no uptake unless measures are taken to enhance the penetration of the cells by MC-LR. Shi et al. (2011) showed that MC-LR could interact with isolated plasma DNA, indicating that cellular uptake may indeed be the limiting factor in the lack of genotoxicity

Table 7- 10. Mutagenicity Assays with Microcystins

Species (test system)	End-point	With metabolic activation	Without metabolic activation	Reference
Ames assay	Gene mutation; Pure MC-LR; extracts containing microcystins	-	-	Ding et al., 1999; Huang et al., 2007
Ames assay	Gene mutation; Crude extract	-	-	Grabow et al., 1982
Ames assay; ara test; SOS/umu test	Gene mutation; MC-LR extract	-	-	Wu et al., 2006
Ames assay	Gene mutation; Purified hepatotoxin assumed to be microcystin	-	-	Repavich et al., 1990
Human embryo fibroblast cells	Gene mutation; MC-LR (purity not specified)	Not applicable	+	Suzuki et al., 1998
Human lymphoblastoid TK6 cells	Gene mutation; 5x increased frequency of thymidine kinase mutations; induction of micronuclei	Not applicable	+	Zhan et al., 2004

in some cell types. No DNA binding and minor groove interactions with MC-LR occurred in this study.

Genotoxicity – in vitro studies

Recent studies suggest that apoptosis may be intimately linked to observations of DNA damage in cells treated with MC-LR. Lankoff et al. (2004a) showed a strong correlation between DNA damage, as measured by the comet assay, and the induction of apoptosis, as measured by the terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick end-labeling (TUNEL) assay, in human lymphocytes. Other evidence has suggested that the comet assay can give a false positive measure of DNA damage when apoptosis is induced, as DNA fragmentation occurs during the process of apoptosis (Lankoff et al., 2004a). The authors postulated that earlier reports of DNA damage measured by the comet assay may have been related to early stages of apoptosis due to cytotoxicity rather than a direct effect on DNA. The induction of apoptosis appears to be dose-related. Humpage and Falconer (1999) showed that low (picomolar) concentrations of commercially-obtained MC-LR induced cytokinesis and inhibited apoptosis in primary mouse hepatocytes, while higher (nanomolar) concentrations resulted in opposite effects. Ding et al. (1999) showed DNA damage in primary rat hepatocytes by the Comet assay at 1 µg MC-LR/mL.

Nong et al. (2007) observed a dose-dependent increase in test tail DNA using the Comet assay in HepG2 cells incubated with 1-100 μM MC-LR (purity not reported) for 24 hours; the 30 and 100 μM concentrations reach statistical significance. Žegura et al. (2006) also found a significant increase in the proportion of tail DNA (indicating DNA damage in the Comet assay) in HepG2 cells incubated with MC-LR (purity not reported) for up to 16 hours. BSO pretreatment increased the susceptibility to MC-LR induced DNA damage, while pretreatment with the glutathione precursor N-acetylcysteine protected against the MC-LR induced DNA damage.

In a study with a similar design using HepG2 cells, Žegura et al. (2008a) observed elevation of p53 and the downregulated genes p21 and gadd45a, which are responsible for cell cycle arrest and DNA repair, as well as mdm2, which is a feedback regulator for p53 expression and activity. The study authors concluded that these findings indicate that MC-LR has genotoxic potential. Žegura et al. (2008b) evaluated the genotoxic effects of MC-LR (purity not reported) on different cell types using the Comet assay. Three human cell lines were used: CaCo-2, which is a human colon adenocarcinoma cell line; IPDDC-A2, which is a human astrocytoma cell line; and NCNC, which is a human B-lymphoblastoid cell line. A significant increase in DNA damage was only observed in CaCo-2 cells. Žegura et al. (2011) observed DNA damage using the Comet assay in human peripheral blood lymphocytes at concentrations of 0.1 to 10 μg/mL of MC-LR (purity not reported). As was previously observed in HepG2 cells, DNA damage-responsive gene p53 was upregulated along with its downstream-regulated genes involved in DNA repair and cell cycle regulation, mdm2, gadd45a, and p21. DNA fragmentation was significantly increased in rat neutrophils with MC-LA and MC-YR, but not in human neutrophils (Kujbida et al., 2008).

Bouaïcha et al. (2005) reported that noncytotoxic concentrations of MC-LR slightly decreased the amount of endogenously formed DNA adducts compared with controls in cultured hepatocytes. MC-LR was shown to cause a dose- and time-dependent increase in the formation of 8-oxo-7, 8-dihydro-2'-deoxyguanosine (a measure of oxidative DNA damage) in cultured hepatocytes (Maatouk et al., 2004; Bouaïcha et al., 2005).

Lankoff et al. (2004a) observed no effect of MC-LR on the incidence of chromosomal aberrations in human peripheral blood lymphocytes. In a separate study by Lankoff et al. (2006a) MC-LR inhibited repair of gamma-induced DNA damage in human lymphocytes and a human glioblastoma cell line.

Observations of polyploidy in MC-LR-treated cells (Humpage and Falconer, 1999; Lankoff et al., 2003) may be related to its effects on cytokinesis. Lankoff et al. (2003) showed that MC-LR, through its effect on microtubules, damages the mitotic spindle, leading to the formation of polyploid cells. Repavich et al. (1990) reported a dose-related increase in chromosome breakage in human lymphocytes exposed to a purified hepatotoxin (presumed to be a microcystin). MC-LR disrupted chromatin condensation in Chinese hamster ovary cells at the end of interphase and the beginning of metaphase (Gácsi et al., 2009).

Neither MC-LR nor cyanobacterial extracts resulted in an increase in micronucleus formation in cultured human lymphocytes (Abramsson-Zetterberg et al., 2010).

Table 7- 11. Genotoxicity of Microcystins *In vitro*

Species (test system)	End-point	Results	Reference
Primary rat hepatocytes	Liver DNA	DNA damage with microcystin extract containing MC-LR.	Ding et al., 1999
Rat hepatocytes	DNA adducts	Noncytotoxic concentrations of MC-LR slightly decreased endogenously formed DNA adducts	Bouaïcha et al., 2005
Rat hepatocytes	DNA adducts	MC-LR caused oxidative DNA adducts	Maatouk et al., 2004; Bouaïcha et al., 2005
Primary mouse hepatocytes	DNA damage	Commercial MC-LR induced cytokinesis and inhibited apoptosis at picomolar concentrations; nanomolar concentrations resulted in inverse effects	Humpage and Falconer, 1999
HepG2 cells	DNA damage	MC-LR increased comet test tail moment.	Nong et al., 2007; Žegura et al., 2006
Human hepatoma cells	Liver DNA and repair	DNA damage with MC-LR; elevated p53 and downregulated p21 and gadd45a	Žegura et al., 2003; 2004; 2008a
CaCo-2, IPDDC-A2, and NCNC human cell lines	DNA damage	MC-LR increased DNA damage only in CaCo-2 cells.	Žetura et al., 2008b
Human and rat neutrophils	DNA damage	MC-LA and MC-YR increased DNA fragmentation in rat, but not human, neutrophils.	Kujbida et al., 2008
Human lymphocytes	DNA damage	MC-LR caused DNA damage and induction of apoptosis but no chromosome aberrations.	Lankoff et al., 2004a
Human lymphocytes and glioblastoma cell line	DNA damage and repair	No micronuclei formation in lymphocytes; inhibited repair of gamma-induced DNA damage.	Lankoff et al., 2006a
Human lymphocytes	DNA damage	MC-LR caused DNA damage and upregulation of damage-responsive genes	Žegura et al., 2011
Human lymphocytes	DNA damage	Dose-related chromosome breakage.	Repavich et al., 1990
Chinese hamster ovary cells	Cell cycle	MC-LR disrupted chromatin condensation.	Gácsi et al., 2009
Human lymphocytes	Micronucleus formation	No increase with MC-LR or extract.	Abramsson-Zetterberg et al., 2011

A number of studies have reported DNA damage after MC-LR treatment *in vivo*. MC-LR was shown to cause a dose- and time-dependent increase in the formation of 8-oxo-7,8-dihydro-2'-deoxyguanosine (a measure of oxidative DNA damage) in rat liver cells after *in vivo* treatment via i.p. injection (Maatouk et al., 2004; Bouaïcha et al., 2005).

Gaudin et al. (2008) found DNA damage in female mice administered MC-LR (>95% pure) via either oral or i.p. injection. Groups of three female Swiss albino mice were administered a single gavage dose of 0, 2, or 4 mg/kg or a single i.p. dose of 10, 25, 40, or 50 µg/kg and sacrificed 3 or 24 hours after treatment. DNA damage was assessed in whole blood, bone marrow, liver, kidney, colon, and intestine using the comet assay. Clinical observations were not reported. After oral administration, a statistically significant dose-dependent increase in DNA damage was observed in blood from both dose groups at three hours, but not at 24 hours; no effects were seen in the other tissues assayed. After i.p. exposure, DNA damage was found at doses ≥40 µg/kg only in bone marrow after 3 hours; after 24 hours DNA damage was found in kidney, intestine and colon at ≥25 µg/kg with the most pronounced effect a dose-related increase in the liver at all doses. In contrast, Gaudin et al. (2009) did not find any DNA damage, as assessed by the Comet assay and unscheduled DNA synthesis, in the livers of female rats administered 12.5-50 µg MC-LR/kg (commercial product; purity not reported) via intravenous injection.

Li et al. (2011b) administered (i.p.) crude extracts from a cyanobacterial bloom containing 244.26 µg MC-LR per gram of lyophilized algae to male mice and observed a dose-dependent increase in olive tail moment from the Comet assay in the liver and testes. MC-YR has also been found to induce DNA damage measured by the Comet assay in the blood (lymphocytes), liver, kidney, lung, spleen, and brain of mice administered 10 µg/kg of MC-YR via i.p. injection every other day for 30 days (Filipič et al., 2007).

Dong et al. (2008) evaluated the genotoxicity of MC-LR (source and purity not provided) in mouse testes. Male KM mice were administered 0, 3, 6, or 12 µg/kg of MC-LR daily for seven days. On day 8, five mice/treatment were sacrificed and their testes were removed for analysis. Five mice per treatment were also sacrificed 14 days after injection to evaluate the micronuclei in the sperm cell early stage. An increase in DNA-protein crosslinks and micronuclei was observed with all doses (no dose response, highest dose lower than mid dose), but statistical significance was reached only at 6 and 12 µg/kg.

Neither MC-LR nor cyanobacterial extracts resulted in an increase in micronucleus formation in erythrocytes from peripheral blood of mice given up to 55 µg/kg (Abramsson-Zetterberg et al., 2010). However, Zhang et al. (2011a) observed a significant increase in the frequency of micronuclei in polychromatic erythrocytes (PCEs) in the bone marrow of rabbits (6/treatment group) administered 6 µg/kg-day microcystin from an extract of *M. aeruginosa* via i.p. injection for 7 or 14 days. The microcystin extracts contained ≥80% total microcystin, with 0.84 mg/g dry weight MC-RR, 0.50 mg/g dry weight MC-LR, and 0.07 mg/g dry weight MC-YR. There was also a significant decrease in PCEs/total erythrocytes. Similarly, dose-related increases in micronuclei formation were seen in bone marrow from male mice given 1-100 mg extract/kg (Ding et al., 1999).

Table 7- 12. Genotoxicity of Microcystins *In vivo*

Species (test system)	End-point	Results	Reference
Mouse	Liver DNA	DNA damage after treatment with MC-LR	Rao and Bhattacharya, 1996
Mouse	DNA damage	MC-LR caused damage in blood cells after oral; in liver, kidney, intestine, and colon after i.p.; none in liver after i.v.	Gaudin et al., 2008; 2009
Mouse	DNA damage	MC-LR extract i.p. caused dose-dependent olive tail moment in liver and testes.	Li et al., 2011b
Mouse	DNA damage	MC-YR given i.p. induced damage in multiple organs.	Filipič et al., 2007
Mouse	DNA damage	Increased DNA-protein crosslinks and micronuclei in testes with MC-LR.	Dong et al., 2008
Rat	Liver DNA	Oxidative damage after i.p. injection of MC-LR	Maatouk et al., 2004; Bouaïcha et al., 2005
Mouse bone marrow erythrocytes	DNA damage	Induction of micronuclei with microcystin extract	Ding et al., 1999
Mouse erythrocytes; peripheral blood	DNA damage	No induction of micronuclei with MC-LR or extract.	Abramsson-Zetterberg et al., 2010
Rabbit bone marrow	DNA damage	Extract containing MC-RR, -LR, and -YR increased frequency of micronuclei in PCEs	Zhang et al., 2011a

7.1.3.2 Tumor Promotion

Mechanistic evidence provides support for the hypothesis that MC-LR can act as a promoter at low doses due to increased cell proliferation and decreased apoptosis, as well as inhibition of repair. Zhu et al. (2005) reported that MC-LR can transform immortalized colorectal crypt cells, resulting in anchorage-independent growth and enhanced proliferation. Lankoff et al. (2006b) did not find any DNA damage in CHO-K1 cells incubated with MC-LR (10 or 24 µg/mL), but MC-LR did inhibit the repair of DNA damage induced by ultraviolet light. The study authors suggested that MC-LR inhibited the nucleotide excision repair through inhibition of the inclusion/exclusion phase as well as the rejoining phase. In a different study, MC-LR inhibited DNA repair by gamma radiation in human lymphocytes and a human glioblastoma cell line (Lankoff et al., 2006a). MC-LR and MC-RR have been shown to increase the expression of the Bcl-2 protein (that inhibits apoptosis) and decrease the expression of the bax protein (that induces apoptosis) (Hu et al., 2002; Hu et al., 2010; Huang et al., 2011; Li et al., 2011d). However, one study found decreased expression of Bax, Bcl-2, and bad (pro-apoptotic)

proteins (Billam et al., 2008). In addition, MC-LR upregulates the transcription factors c-fos and c-jun, leading to abnormal proliferation (Zhao and Zhu, 2003; Li et al., 2009).

Gehringer (2004) reviewed the molecular mechanisms leading to promotion by MC-LR and the related tumor promoter, okadaic acid. Gehringer (2004) reported that MC-LR inhibits protein phosphatase PP2A, which regulates several mitogen-activated protein kinases (MAPK). The MAPK cascade regulates transcription of genes required for cell proliferation, including c-jun and c-fos. Activation of the MAPK cascade has also been postulated to inhibit apoptosis and thus increase cell proliferation. Finally, Gehringer (2004) noted that MC-LR has been reported to increase phosphorylation of p53, which is involved in the regulation of the cell cycle and apoptosis as well as the carcinogenic process. Zhang et al. (2010) found increased MAPK phosphorylation in the livers of mice orally exposed to MC-LR.

Xing et al. (2008) observed increases in p53 expression and decreased PP2A expression in FL human amniotic epithelial cells incubated with 10-1000 nM MC-LR for 24 hours. Hu et al. (2008) observed a significant increase in p53 expression in livers of rats exposed to pure MC-LR (purity not reported) via i.p. injection twice a week for 6 weeks, but did not observe a significant increase in p53 expression with cyanobacterial extracts containing MC-LR at a concentration of 529.656 ng/L administered via the drinking water. Neither treatment altered p16 expression. Takumi et al. (2010) studied the role of p53 on cell fate in HEK293-OATP1B3 cells exposed to MC-LR. The data suggested that when p53 is inactivated, chronic low exposure to MC-LR could lead to cell proliferation through activation of Akt signaling. Akt is a general mediator of growth factor induced survival and has been shown to suppress the apoptotic death of a number of cell types induced by a variety of stimuli, including growth factor withdrawal, cell-cycle discordance, loss of cell adhesion, and DNA damage

Clark et al. (2007, 2008) found MC-LR administered i.p. at a sublethal dose caused changes in gene transcription related to actin organization, cell cycle, apoptotic, cellular redox status, cell signaling, albumin metabolism, and glucose homeostasis pathways, as well as the OAT_P system in the livers of p53 knockout mice. The gene expression analysis found increases in genes related to cell-cycle regulation and cellular proliferation in MC-LR treated mice livers compared to the p53 deficient control livers, that was greater than that observed in the livers of MC-LR treated wild type mice (Clark et al., 2008). Ki-67 (a marker of cell proliferation) and phospho-histone H3 (a mitotic marker) for immunoreactivity were also increased in MC-LR-treated knockout mice. The study authors concluded that p53 may play an important role in tumor promotion by MC-LR.

Assays on microRNA expression in WRL-68 cells revealed MC-LR (10 μg/L for 25 passages) induced alterations in two onco-microRNAs, miR-21 and miR-221 (Xu et al., 2012). Both were upregulated, while liver-specific miR-122 was down regulated. Subcutaneous injection of these cells into nude mice was associated with tumor development in all six mice used, while no tumors developed at control sites. Fu et al. (2009) also found changes in proteins associated with the cell cycle in human amniotic epithelial cells exposed to MC-RR.

Changes in MMP levels have been linked to cancer and tumor promotion. Zhang et al. (2010; 2012) found increased levels of MMP2 and MMP9 in the livers of male mice orally

administered MC-LR for at least 180 days (subchronic and chronic results of these studies were described in Sections 7.1.2.3 and 7.1.2.6, respectively).

7.1.3.3 Immunotoxicity

Shirai et al. (1986) reported that mice, immunized i.p. with either live or sonicated cells from a *Microcystis* water bloom, developed delayed-type hypersensitivity when challenged 2 weeks later with a subcutaneous injection of sonicated cells. Delayed hypersensitivity was assessed by footpad swelling, which was increased approximately 2-fold over controls at the highest doses of cells. It is not clear whether an endotoxin in the blood sample was responsible for the development of hypersensitivity, or whether the antigenic epitope existed on other components of the sample.

Shen et al. (2003) assessed the effect of cyanobacterial cell extract on immune function. Mice received 14 daily i.p. injections containing a cell-free extract from a water bloom dominated by *M. aeruginosa*. Doses were reported as 16, 32 and 64 mg lyophilized cells/kg body weight or as 4.97, 9.94 and 19.88 μg/kg of microcystin equivalents. HPLC analysis indicated that the microcystin content of the extract was 79.53%, although specific congeners in the extract were not reported. The following immunotoxicity endpoints were examined: phagocytosis, lymphocyte proliferation and antibody production in response to sheep red blood cells. Phagocytic capacity was reduced at the two highest doses, but percentage phagocytosis was not affected. B-lymphocyte proliferation was significantly reduced (33% compared to controls at 32 mg/kg), while changes in T-lymphocyte proliferation were mild, and deemed biologically insignificant. Finally, humoral immune response, as measured by antibody-forming plaques, was reduced in a dose-dependent manner in treated mice. Body weight was also significantly reduced in all treatment groups. Relative spleen weight was significantly increased at 9.94 μg/kg, but was significantly decreased at 19.88 μg/kg. Relative thymus weight was significantly decreased in the high-dose group. Relative liver weight was significantly increased in all treatment groups, but was not related to dose.

Several studies have evaluated the effects of MC-LR on immune system components *in vitro* (Lankoff et al., 2004b; Teneva et al., 2005; Chen et al., 2005a; Kujbida et al., 2006). Lankoff et al. (2004b) reported that MC-LR inhibited B-cell proliferation in human and chicken peripheral blood lymphocytes at all concentrations tested and decreased T-cell proliferation only at the highest concentration. Apoptosis was enhanced in both human and chicken lymphocytes (Lankoff et al., 2004b). Similarly, MC-LR was cytotoxic to mouse splenocytes, and caused apoptosis in B-cells but not in T-cells (Teneva et al., 2005).

Kujbida et al. (2006) assessed the effects of MC-LR and [Asp3]-MC-LR on human polymorphonuclear lymphocytes (PMNs) *in vitro*. Both compounds caused migration of neutrophils in a chemotaxis chamber, suggesting that PMNs may migrate from the blood stream to organs that concentrate microcystins, such as the liver. In addition, both caused a dose-related increase in reactive oxygen species (ROS) production as measured by chemiluminescence of PMN degranulation products that accompany ROS production. The phagocytosis of *Candida albicans* by PMNs was increased after exposure to either compound, but only MC-LR increased the intracellular killing of *C. albicans*. These findings suggest the possibility that PMNs may

mediate some of the toxic effects of microcystins. Kujbida et al. (2008) also found that MC-LR, MC-LA, and MC-YR increased interleukin-8 levels and extracellular ROS in human neutrophils, and chemoattractant-2αβ in rat neutrophils, but had no effect on tumor necrosis factor-α in either rat or human neutrophils. *In vitro* all three microcystins caused neutrophil chemotaxis by increased intracellular calcium levels (Kujbida et al., 2009). *In vivo,* topical application of MC-LR to male rats caused an enhancement of the number of rolling and adhered leukocytes in the endothelium of postcapillary mesenteric venules, but MC-LA and MC-YR had no effect (Kujbida et al. 2009).

Shi et al., 2004 reported a study where mice received a single i.p. injection containing a cell-free extract from a water bloom dominated by *M. aeruginosa* processed in the same manner as the Shen et al. (2003) study. Although specific congeners in the extract were not reported, it was stated that MC-LR was the predominant component. Doses were reported as 0, 23, 38, 77 and 115 mg lyophilized cells/kg body weight or as 0, 7, 12, 24 and 36 µg/kg of microcystin equivalents. Animals were sacrificed 8 hours after exposure. Messenger RNA levels of TNF-α, IL-1β, IL-2, and IL-4 were significantly decreased, IL-6 was unaffected, and IL-10 was increased at the lowest dose and decreased at higher doses. None of the changes were dose-related.

Chen et al. (2004b, 2005a) evaluated the role of nitric oxide generation and macrophage related cytokines on the reduced phagocytic capacity induced by pure MC-LR. A dose-dependent inhibition of nitric oxide production was observed in activated macrophages, and a repressive effect was seen in cytokine formation at the mRNA level (e.g., IL-1β, TNF-α, GM-CSF, IFN-γ) after either a 24-hour (Chen et al., 2004b) or a 6-hour treatment (Chen et al., 2005a). Hernandez et al. (2000) indicated that MC-LR enhanced the early spontaneous polymorphonuclear leukocyte (PMN) adherence (not late or PMN stimulated early or late) at low concentrations, suggesting that microcystins may affect the immune system.

Yuan et al. (2012) used extracts of microcystins isolated from a surface bloom in China to evaluate immunotoxicity in rabbits (4/treatment group). The extracts contained MC-RR, MC-LR, and MC-YR at the following concentrations: 0.84, 0.50, and 0.07 mg/g dry weight, respectively. Rabbits were administered 0, 12.5, or 50 µg MC-LR equivalents/kg via a single i.p. injection. Sera was collected from the heart at 0, 1, and 3 hours after administration of the 50 µg/kg dose and 0, 1, 3, 12, 24, 48, and 168 hours after administration of the 12.5 µg/kg dose. There was a significant increase in plasma white blood cells after MC-LR treatment with both doses. Peak increase was observed 1 hour after 50 µg/kg and 12 hours after 12.5 µg/kg. After 50 µg/kg, interferon (IFN)-γ, INF-α, IL-3, IL-4, and IL-6 production was decreased at all time points measured. However, at the 12.5 µg/kg dose, production of interferon (IFN)-γ, INF-α, IL-3, IL-4, and IL-6 was increased through 12 hours after exposure, but was lower or similar to the controls from 24 to 168 hours.

7.1.3.4 Hematological Effects

Several studies have noted thrombocytopenia (platelet deficiency) in laboratory animals treated with microcystins or bloom extracts purportedly containing microcystins (Slatkin et al., 1983; Adams et al., 1985, 1988; Takahashi et al., 1995). Early investigations explored whether

microcystins had a direct effect on platelets, and whether platelets might be responsible for pulmonary thrombi (Slatkin et al., 1983; Jones, 1984). However, *in vitro* studies have shown that MC-LR neither induces nor impedes the aggregation of platelets (Adams et al., 1985). Pulmonary thrombi apparently consist of necrotic hepatocytes circulating in the blood. More recent information supports the hypothesis that hematological effects observed in animals acutely exposed to microcystins are secondary effects of liver hemorrhage (Takahashi et al., 1995).

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Takahashi et al. (1995) reported dose-dependent reductions in erythrocyte count, leukocyte count, hemoglobin concentration,hematocrit and coagulation parameters 1 hour after rats were exposed to MC-LR (100 and 200 µg/kg i.p). None of these parameters changed until after massive liver hemorrhage commenced. Further, hematological changes such as increased prothrombin time and fibrin deposition in the renal glomeruli were not observed. The authors concluded that the depletion of blood components occurred as a result of liver hemorrhage.

Sicińska et al. (2006) evaluated the effects of MC-LR on human erythrocytes *in vitro.* MC-LR exposure resulted in the formation of echinocytes, hemolysis, conversion of oxyhemoglobin to methemoglobin, and a decrease in membrane fluidity. In addition, measures of oxidative stress were affected in treated erythrocytes; glutathione reductase and superoxide dismutase activities were decreased, while ROS and lipid peroxidation were increased.

7.1.3.5 Physiological or Mechanistic Studies

7.1.3.5.1 Noncancer Effects

Many mechanistic studies have been conducted to characterize the toxicology of microcystins. These studies include *in vivo* investigations in laboratory animals, *in situ* studies in isolated perfused organ systems and *in vitro* assays in isolated cell preparations. Mechanistic studies have evaluated many aspects of microcystin toxicity, including: 1) interaction with serine and threonine protein phosphatases (i.e., PP1 and PP2A) as the molecular target for microcystins, 2) the role of cytoskeletal effects, 3) apoptosis, 4) the importance of oxidative stress as a mode of toxic action, and 5) the reasons for target organ and cell type specificity of microcystins. Each of these topics is discussed in further detail below.

Protein Phosphatase Inhibition

The primary molecular target of microcystins has been identified as the protein phosphatase enzymes PP1 and PP2A. Protein phosphatases function in the post-translational modification of phosphorylated cellular polypeptides or proteins. PP1 and PP2A groupings belong to the PPP family of protein phosphatases which hydrolyze the ester linkage of serine and threonine phosphate esters. Both enzyme groupings have a single catalytic unit which is joined to a variety of regulatory and targeting subunits. In higher eukaryotes there are approximately 1,000 protein phosphatase genes which confers considerable regulatory diversity to the individual superfamilies (Barford et al., 1998)

The actions of members of the protein kinase family of enzymes precede that of the protein phosphatases because they esterify phosphates to the hydroxyl functional groups of serine, threonine and tyrosine in proteins. Together, kinases and phosphatases maintain the balance of phosphorylation and dephosphorylation for key cellular proteins involved in a variety of activities including metabolic processes, gene regulation, cell cycle control, transport and secretory processes, the organization of the cytoskeleton, and cell adhesion (Barford et al., 1998).

The molecular interaction between microcystins and protein phosphatases has been evaluated using immunoprecipitation, autoradiography, reverse phase liquid chromatography, X-ray crystallography, nuclear magnetic resonance (NMR) solution structures, and molecular dynamics simulation (Runnegar et al., 1995b; MacKintosh et al., 1995; Goldberg et al., 1995; Craig et al., 1996; Bagu et al., 1997; Mattila et al., 2000; Mikhailov et al., 2003; Maynes et al., 2004, 2006). Molecular modeling and molecular dynamics simulations have indicated that microcystins bind in a Y-shaped groove containing the catalytic site on the surface of PP1 (Mattila et al., 2000). Studies with PP1 suggest that the C-terminal β12-β13 loop of PP1 (containing residues 268-281) is important for microcystin-protein phosphatase interactions as well as for substrate recognition (Maynes et al., 2004, 2006). Information available to date indicates that the binding process primarily involves the amino acids glutamate, Adda, leucine and Mdha of microcystins.

Microcystins LR, LA and LL interact with the catalytic subunits of PP1 and PP2A in two phases. The first phase occurs within minutes and consists of rapid inactivation of the phosphatase. The second, slower phase of interaction represents a covalent interaction that takes place within several hours (Craig et al., 1996). The initial binding and inactivation of protein phosphatases appears to result from several non-covalent interactions that are still being elucidated. Mattila et al. (2000) demonstrated an interaction of the glutamate-free carboxylate of MC-LR with a metal ion (Fe or Mn) in the PP1 catalytic site. Glutamate appears to be an important component because esterification of the carboxylate functional group eliminates toxicity (Namikoshi et al., 1993; Rinehart et al., 1994). Herfindal and Selheim (2006), in a review of the mechanisms of microcystin toxicity, indicated that the Adda side chain is involved in a hydrophobic interaction between the tryptophan 206 and isoleucine 130 residues in the hydrophobic groove of PP1.

Mattila et al. (2000) suggested that the long hydrophobic side chain of the Adda residue may contribute to orienting the toxin into the hydrophobic groove of the catalytic site. The Adda amino acid residue of microcystins plays an important role in the inhibition of protein phosphatase activity (Nishiwaki-Matsushima et al., 1991; Gulledge et al., 2002, 2003a,b). Isomerization of the diene from 4E,6E to 4E,6Z on the Adda chain eliminates the toxic activity of microcystins (Harada et al., 1990; Nishiwaki-Matsushima et al., 1991; Stotts et al., 1993). Microcystin analogues containing only Adda and one additional amino acid are capable of substantial inhibition of PP1 and PP2A, while modifications to the Adda structure abolished the inhibition (Gulledge et al., 2003b). Herfindal and Selheim (2006) indicated that the L-Leucine of MC-LR participates in a hydrophobic interaction with Tyrosine 272 of PP1 (on the β12-β13 loop).

The second phase of interaction between microcystins and protein phosphatase consists of covalent bonding (Craig et al., 1996). Immunoprecipitation and autoradiography methods indicate that a covalent bond results from the interaction between the methylene of the Mdha residue of microcystins and the thiol of Cys273 residue of PP1. NMR solution structures and X-ray crystallography data on the MC-LR/PP1 complex illustrate the covalent linkage at Cys273 (Goldberg et al., 1995; Bagu et al., 1997). Site-directed mutagenesis replacing Cys273 in PP1 results in a loss of microcystin binding (MacKintosh et al., 1995; Maynes et al., 2004). Based on sequence similarity between PP1 and PP2A, it has been suggested that Cys-266 is the site of a covalent linkage between PP2A and microcystins (Craig et al., 1996).

Microcystin analogues containing a reduced Mdha residue are not capable of covalent binding to protein phosphatases. MacKintosh et al. (1995) reported that a modification of the Mdha residue of MC-YR by reaction with ethanethiol abolished covalent binding to PP1. Likewise, Craig et al. (1996) showed that reduction of the Mdha residue of MC-LA with $NaBH_2$ abolished the covalent binding phase with PP2A. Maynes et al. (2006) confirmed the lack of covalent interaction by determining the crystal structure of dihydroMC-LA bound to PP1. Their work showed that the β12-β13 loop of PP1 takes on a different conformation when the covalent bond is absent, and that other interactions (including hydrogen bonding) are responsible for the bond between dihydroMC-LA and PP1.

The relevance of covalent bonding between microcystins and protein phosphatases to enzyme inhibition is uncertain, as other interactions are apparently responsible for the rapid inactivation of the enzymes (Herfindal and Selheim, 2006). Modifications to either molecule (microcystin or protein phosphatase) to prevent covalent bonding generally decrease, but do not eliminate, the toxic action (Meriluoto et al., 1990; MacKintosh et al., 1995; Hastie et al., 2005).

Microcystins bind to the phosphatase enzymes under both *in vivo* and *in vitro* conditions, resulting in an inhibition of enzyme activity leading to a decrease in protein dephosphorylation. Microcystins have been shown to directly inhibit the activity of PP1 and PP2A derived from different species (i.e., fish, mammals, plants) and cell types (cultured cell lines as well as isolated tissue cells) (Honkanen et al., 1990; MacKintosh et al., 1990; Matshushima et al., 1990; Yoshizawa et al., 1990; Sim and Mudge, 1993; Xu et al., 2000; Leiers et al., 2000; Becchetti et al., 2002). Microcystin has also been found to bind to PP4, another member of the protein phosphatase family (Imanishi and Harada, 2004

Ito et al. (2002b) observed a similar degree of inhibition of protein phosphatases 1 and 2A *in vitro* with MC-LR and its glutathione and cysteine conjugates. However, Metcalf et al. (2000) demonstrated weaker inhibition of PP1 and PP2A *in vitro* by microcystin glutathione, cysteine-glycine, and cysteine conjugates than by parent microcystins; these conjugates also are less toxic in the mouse bioassay than parent microcystin. As noted in Section 6.3.1, Kondo et al. (1992, 1996) postulated that the Adda and Mdha moieties could be the sites of CYP oxidation and subsequent conjugation with glutathione or cysteine.

Microcystins have been used as a tool to investigate the importance of serine and threonine phosphorylation to specific cellular functions. The regulatory effects of phosphorylation on the sodium channel increases the probability of the channel being open in

renal cells (Becchetti et al., 2002). Phosphorylation appears to inhibit ATP-dependent actin and myosin interaction in smooth and skeletal muscle contraction (Hayakawa and Kohama, 1995; Knapp et al., 2002), and increase insulin secretion (Leiers et al., 2000).

Several *in vitro* studies indicate that low levels of microcystins can upregulate protein phosphatase mRNA expression such that protein phosphatase activity may be increased rather than decreased. Liang et al. (2011) used the FL amniotic epithelial cell line to test the effects of MC-LR and found that incubation for 6 hours with low concentrations (0.5 or 1 μM) caused increases in PP2A activity, while incubation for 24 hours with higher concentrations (i.e., 5 or 10 μM) caused a decrease in PP2A activity. They discovered that the increases in PP2A activity were due to the up-regulation of mRNA and protein levels of the C subunit. Fu et al. (2009) and Xing et al. (2008) found comparable upregulation of PP2A in FL cells at comparable concentrations and incubation times. However, Huang et al. (2011) did not find any change in PP2A activity or PP2A A subunit expression in the livers of male mice after 7 days of oral exposure with doses up to 186 μg MC-RR/kg.

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Li et al. (2011c) tested the effects of MC-LR on PP2A in human embryonic kidney (HEK) 293 cells. PP2A activity was inhibited with concentrations of 5-10 μM (only significantly inhibited with 7.5 and 10 μM), increased at concentrations of ≤2.5 μM (only statistically significant with 1 and 2 μM). Treatment with MC-LR caused a disassociation between PP2A and its α4 regulatory subunit, at all doses tested. The study authors suggested that disassociation of α4, a PP2a subunit that regulates activity of PP2A leading to an increase in active PP2A catalytic subunit in the cell, could explain the higher activity at low concentrations. At higher concentrations the increase in the PP2A catalytic unit is unable to compete with the inhibitory effects of MC-LR.

Not all microcystins are equipotent inhibitors of protein phosphatases. Table 7-13 provides comparative data of the IC_{50} values for inhibition of protein phosphatases (IC_{50}s) by MC-LR, MC-YR, MC-RR and MC-LA as reported by several different authors. The Table demonstrates that there is not much consistency in the results. Differences across studies are likely due to the conditions of the individual studies. There is also a lack of consistency in the relative potencies of individual microcystins across the individual studies.

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Table 7- 13. Studies Comparing Protein Phosphatase Inhibition Activity of Microcystin Congeners

Reference	IC_{50} (nM)			
	MC-LR	MC-LA	MC-YR	MC-RR
PP2A Inhibition				
Craig et al., 1996	0.15	0.16		
Nishiwaki-Matsushima et al., 1991	0.28			0.78
Matsushima et al., 1990	7.6		4.5	5.8
PP1 Inhibition				
MacKintosh et al., 1995	0.2		0.2	
Mixture of PPs				
Yoshizawa et al., 1990	1.6		1.4	3.4

Cytoskeletal Disruption

Protein phosphatase inhibition by microcystins relates to changes in cytoskeletal structure and cell morphology (Eriksson and Golman, 1993). The cytoskeleton is comprised of a variety of polymeric, proteinaceous filaments that form a flexible framework for the cell. The cytoskeleton provides attachment points for organelles within cells, and makes communication between parts of the cell and between cells possible (Sun et al., 2011). The major cytoskeletal proteins can be broadly categorized (Hao et al., 2010) as microfilament proteins (e.g. actins and myosin; 7 nm diameter), intermediate filaments (e.g. keratins, desmins; 10 nm diameter), and microtubules (e.g. dyneins, tubulin; 25 nm diameter). In addition, there are a broad number of individual proteins that are associated with the microtubules and microfilaments. Serine-threonine proteases are of critical importance in maintaining cytoskeletal integrity (Erickson et al., 1992 a,b) because of their dephosphorylating impact on phosphoprotein-cytoskeletal precursors.

The cytoskeletal effects of microcystins in the liver have been visually demonstrated in several studies using light, electron and fluorescent microscopy (Runnegar and Falconer, 1986; Eriksson et al., 1989; Hooser et al., 1989a,b, 1991b; Falconer and Yeung, 1992). Ultrastructural changes in rats given a lethal dose of microcystin-A include the following:

- a widening of intracellular spaces;
- progressive cell-cell disassociation followed by rounding, blebbing and invagination of hepatocytes;
- loss of microvilli in the space between the hepatocytes and sinusoids;
- breakdown of the endothelium;
- hemorrhage; and
- loss of lobular architecture (Hooser et al., 1989b).

No effects were noted in liver endothelial cells or Kupffer cells. In other studies of isolated hepatocytes, actin aggregates were seen at the base of the membrane blebs following microcystin exposure. As membrane blebs grew larger and were drawn toward one pole of the cell, the microfilaments were organized toward the same pole, resulting in rosette formation with a condensed band of microfilaments at the center (Runnegar and Falconer, 1986; Eriksson et al., 1989b; Hooser et al., 1991b; Falconer and Yeung, 1992; Wickstrom et al., 1995; Ding et al., 2000a).

Similar histopathological changes in the rat testes have been described by Chen et al. (2013). Repeated i.p. dosing resulted in increased space between the seminiferous tubules, cytoplasmic shrinkage, cell membrane blebbing, swollen mitochondria, and deformed nuclei. Transcriptional levels of β-actin and β-tubulin were significantly decreased.

Studies in primary isolated hepatocytes have described the morphological and histopathological changes induced by microcystins that relate to loss of sinusoidal architecture and cytotoxicity. Microcystin exposure to hepatocytes in suspension or cultured in a monolayer results in membrane blebbing that becomes more pronounced and localized in one region of the cell surface. Cells are observed to be rounded in appearance and become dissociated from one another. Microfilaments are reorganized as a compact spherical body in the vicinity of the

blebbing, while the rest of cell is depleted of filamentous actin. MC-LR disrupts hepatocellular morphology within minutes, leading to loss of sinusoidal architecture and hemorrhage. Morphological changes in hepatocytes (i.e., blebbing, rounding) occurred prior to any effect on cell membrane integrity (measured as LDH leakage or release of radiolabeled adenine nucleotides) or cell viability (generally measured as decreased trypan blue exclusion) (Runnegar et al., 1981; Runnegar and Falconer, 1982; Aune and Berg, 1986; Ding et al., 2000a).

Thompson et al. (1988) described the time course of cellular effects of microcystins (type not specified) on primary cultures of rat hepatocytes. Cells were isolated, attached in a monolayer, treated with 0.001-10 µg/mL of microcystin, and monitored for 24 hours. Disintegration of attachment matrix occurred by 15 minutes at the highest concentration. At one hour, cells clustered in groups with no extracellular material. Between 2 and 4 hours cells began to release from the plates. LDH release did not occur until after these visual effects, but when present, was concentration-related.

The observed reorganization of microfilaments leading to alteration of hepatocyte morphology does not appear to be due to effects on actin polymerization (Runnegar and Falconer, 1986; Eriksson et al., 1989b; Falconer and Yeung, 1992). Instead, microcystins caused a decrease in the dephosphorylation of cytokeratin intermediate filament proteins (Falconer and Yeung, 1992; Ohta et al., 1992; Wickstrom et al., 1995; Blankson et al., 2000). Toivola et al. (1997) evaluated the effects of MC-LR on hepatic keratin intermediate filaments in primary hepatocyte cultures. A disruption of the desmoplakin, a cytoskeletal linker protein that connects an intermediate filament to the plasma membrane, was followed by a dramatic reorganization of the intermediate filament and microfilament networks, resulting in intermediate filaments being organized around a condensed actin core.

The major target proteins for microcystin-induced hyperphosphorylation include keratins 8 and 18 and desmoplakin I/II. Keratins 8 and 18 are the major proteins of intermediate filaments in hepatocytes; desmoplakin I and II attach keratin filaments in epithelial cells to desmosomes, (complexes of adhesions proteins that function in cell to cell adhesion). Hyperphosphorylation of desmoplakin I/II leads to loosening of cell junction and loss of interactions with cytoplasmic intermediate filaments. The hyperphosphorylation of keratin proteins prevents subunit polymerization leading to the observed morphological changes. A Ca^{2+}/ calmodulin-dependent kinase may be involved in regulating the serine-specific phosphorylation of keratin proteins 8 and 18. Kinase-induced phosphorylation in the absence of phosphatase dephosphorylation results in the disassembly of the microfilaments, breakdown of the cytoskeleton and its anchoring to desmoplankin I and II (Toivola et al., 1998).

The cell-type specificity of microcystins was investigated using isolated rat hepatocytes, rat renal epithelial cells (ATCC 1571) and rat skin fibroblasts (ATCC 1213) (Khan et al., 1995; Wickstrom et al., 1995). The time course of light microscopic and ultrastructural effects was examined following *in vitro* exposure to MC-LR (Khan et al., 1995). Effects were noted after 4 minutes in hepatocytes, 1 hour in renal cells and 8 hours in fibroblasts. Similar lesions observed in all cell types included blebbing, loss of cell-cell contact, clumping and rounding, cytoplasmic vacuolization and redistribution of cellular organelles. Effects that were seen only in hepatocytes

include loss of microvilli, whirling of rough ER, dense staining and dilated cristae of mitochondria plus pinching off of membrane blebs.

Meng et al. (2011) demonstrated that MC-LR causes reorganization of the cytoskeletal structure in the neuroendocrine PC12 cell line. Pretreatment with a p38 MAPK inhibitor blocked the cytoskeletal alterations as well as the hyperphosphorylation of tau and HSP27. Direct PP2A inhibition by MC-LR and indirect p38 MAPK activation may be responsible for the hyperphosphorylation of tau and HSP27 causing cytoskeletal disorganization.

In addition, Meng et al. (2011) evaluated the effects of microcystin-LR on cultures of a human liver cell line (HL7702). As was the case for the PC12 cell line, hyperphosphorylation of Heat Shock Protein 27 in the presence of microcystin as a phosphatase inhibitor was accompanied by increased activity of several kinases (p38 MAPK, JNK and ERK1/2) leading to cytoskeleton reorganization. Treatment with kinase inhibitors reduced the cytoskeletal changes (Sun et al., 2011). Taken together these studies implicate kinase-induced phosphorylation combined with inhibition of phosphatase removal of key phosphate moieties from serine or threonine esters as the cause of the cytoskeletal changes. When microcystins are present, balance can be partially restored by inhibiting the kinases.

Effects of MC-LR on transcription of cytoskeletal genes of rats exposed intravenously to MC-LR (purified from a bloom) were reported by Hao et al. (2010). Alterations in transcription of genes for actin, tubulin, an intermediate filament (vimentin), and six associated proteins (erzin, radixin, moesin, MAP1b, tau and stathmin) were seen in the liver, kidney, and spleen. Ezrin, moesin, and stathmin are tumor-associated genes which may contribute to tumor promotion by microcystins.

The direction and degree of the cytoskeletal protein change depended on time of measurement after exposure and the organ examined. Effects were most pronounced in the liver. While there were numerous changes that occurred in the transcription of the nine cytoskeletal genes, only a few of the changes were directly correlated with the levels of microcystin in the tissue. Alterations in the transcription of actin (increased), tau (decreased), ezrin (increased), and radixin (increased) in the liver were correlated with tissue microcystin levels (MC-LR, MC-RR, and total levels). Other apparent trends included a steady increase in vimentin and MAP1b in the liver over time, followed by progressively lower levels. The levels of tubulin and stathmin in the liver ended up below control levels.

As explained above, the responses in the liver differed from those in other tissues. In the kidney, increased transcription of stathmin was significantly correlated with levels of MC-RR. In the spleen, a decrease in transcription of radixin was significantly correlated with the levels of MC-RR or total microcystin. The levels of actin at the time of the final measurement were lower than the control in both the kidney and the spleen.

Apoptosis

The ultrastructural changes observed in hepatocytes after microcystin exposure suggest that cell death is related to apoptosis and not necrosis. These changes include cell shrinkage

(decreased volume and increased density), condensation of chromatin and segregation of organelles separated by apoptotic microbodies presumably related to the cytoskeletal damage discussed in the previous section (Boe et al., 1991; Fladmark et al., 1998; McDermott et al., 1998; Ding et al., 2000b; Mankiewicz et al., 2001). The effects of microcystins on signaling pathways involved in rapid apoptosis have been investigated in several studies (Ding et al., 1998a,b, 2000b, 2001, 2002; Ding and Ong, 2003; Huang et al., 2011; Feng et al., 2011; Ji et al., 2011).

In an abstract of a foreign publication by Lei et al. (2006), rates of apoptosis were approximately 22-29% in L-02 cells (a hepatic cell line) incubated with different concentrations of MC-LR for 36 hours, but were increased to 80% after 60 hours of treatment with 50 µg/mL of MC-LR. ROS levels were also seen to increase in a time-dependent manner from 0.5-12 hours in male mice administered a single i.p. injection of 55 µg MC-LR/kg (purity not reported). In male mice orally exposed to MC-RR, apoptosis occurred in the liver (Huang et al., 2011). Changes in protein expression including decreased Bcl-2 (an antiapoptotic regulator) and increased Bax (a proapoptotic regulator), resulted in a significant increase in the ratio of Bax/Bcl-2. These changes are indicative of altered regulation of the outer mitochondrial membrane apoptosis channel proteins (Campos and Vasconcelos, 2010).

Commented [IS48]: While presumably unintentional, the term "foreign publication" reads to be just a little condescending here. Try "non-English publication, or perhaps "Chinese language publication" for this particular citation.

Botha et al. (2004) demonstrated that apoptosis and oxidative stress can be induced in nonhepatic cells by microcystins. MC-RR changed the concentration of several proteins associated with apoptosis in FL human amniotic epithelial cells (Fu et al., 2009). LDH leakage and increased apoptotic indices were observed in the human colon carcinoma cell line (CaCo2) and MCF-7 cells (deficient in pro-caspase-3). These changes were accompanied by increased H_2O_2 formation and increased calpain activity. Chen et al. (2011) found an increase in apoptosis in testes cells in male mice orally administered low doses of MC-LR. Zhang et al. (2011b) also observed apoptosis in Sertoli cells isolated from rats incubated with 10 µg/mL of MC-LR for 24 hours. Accompanying this were increases in p53, Bax, and caspase-3, and a decrease in Bcl-2. Gácsi et al. (2009) observed a dose-dependent increase in apoptosis in Chinese hamster ovary cells through 48 hours of exposure to MC-LR. Apoptosis also occurred *in vitro* with a rat insulinoma cell line exposed to MC-LR for 72 hours (Ji et al., 2011).

Microcystins have been shown to increase expression of p53, plus the pro-apoptotic Bax and Bid proteins and decrease expression of the anti-apoptotic Bcl-2 protein in both *in vitro* and *in vivo* studies (Fu et al., 2005; Weng et al., 2007; Xing et al., 2008; Takumi et al., 2010; Huang et al., 2011; Li et al., 2011d) and mRNA (Lei et al., 2006; Zegura et al., 2008a; Qin et al., 2010; Zegura et al., 2011; Li et al., 2011d). The same concentrations of MC-LR that induced apoptosis in HepG2 cells were found to induce Fas receptor and Fas ligand expression (a critical step in inducing apoptosis) at both the protein and mRNA level (Feng et al., 2011). In addition, MC-LR induced nuclear translocation and activation of the p65 subunit of NF-κB, a signal transduction protein that controls a number of cellular processes including many linked to inflammation and apoptosis (Feng et al., 2011). Knock-down of p65 in HepG2 cells caused a reduction in MC-LR-induced Fas receptor and Fas ligand expression and reduced apoptosis. These findings suggest that MC-LR-induced apoptosis is a complex process involving many cellular signaling proteins.

Opening of the mitochondrial permeability transition (MPT) pores increasing permeability is considered to be a critical rate-limiting event in apoptosis. An early surge of mitochondrial Ca^{2+} was shown to occur prior to MPT and cell death. Prevention of this Ca^{2+} surge by one of several methods (i.e., chelation of intracellular Ca^{2+}, blockage of the mitochondrial Ca^{2+} uniporter or use of an mitochondrial uncoupler) prevented MPT and cell death. Electron transport chain inhibitors (e.g., rotenone, actinomycin A, oligomycin or carbonyl cyanide m-chlorophenylhydrazone) also inhibited the onset of MPT. MC-LR caused the release of cytochrome c through MPT, which is considered as a universal step in mitochondrial apoptosis; however, caspases-9 and -3 which are also linked to apoptosis were not activated. The increase in intracellular Ca^{2+} may instead facilitate the activation of calpain, a calcium-dependant protease, following exposure to microcystins (Ding and Ong, 2003).

In an English abstract from a foreign publication by Liu et al. (2011), i.p. administration of 50 µg MC-LR/kg to mice caused an increase in ALT, AST, Bcl-2 protein, and liver ROS levels; a decrease in mitochondria membrane potential; and a significant DNA ladder indicative of apoptosis. Administration of a MPT inhibitor, cyclsporin A, 1 hour before injection of MC-LR blocked the effects. Thus, inhibiting MPT inhibited MC-LR-induced apoptosis. Mitochondrial respiration was decreased in primary hepatocytes and isolated kidney mitochondria incubated with MC-LR (Jasionik et al., 2010; La-Salete et al., 2008). An uncoupling effect on the mitochondria was observed in both studies, as well as an indication of mitochondrial-generated ROS.

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A role for the endoplasmic reticulum stress pathway is also implicated in MC-LR-induced liver apoptosis in male ICR mice treated i.p. with 20 µg/kg (≥ 95% pure) in a study by Qin et al. (2010). The study measured mRNA and protein levels of endoplasmic reticulum stress-specific molecules in the liver and kidney. Increases in mRNA and protein expression of CHOP (an apoptosis linked protein) and cleaved capase-12 were observed in the liver where apoptotic cells also were noted. There was slight inhibition of these proteins in the kidney where no apoptosis was observed. Bcl-2 was down-regulated in the liver and slightly up-regulated in the kidney. Xing et al. (2008) also observed regulation of CHOP in cells incubated with MC-LR for 24 hours.

Reactive Oxygen Generation Cellular Response

Oxidative stress may play a role in the induction of MPT and the onset of apoptosis. In cultured hepatocytes exposed to microcystins, an increase in the generation of ROS preceded the onset of MPT, mitochondrial depolarization and apoptosis. A dose- and time-dependent increase in ROS and lipid peroxidation, measured as malondialdehyde formation, was shown to precede morphological changes in hepatocytes and release of LDH. The addition of deferoxamine or cyclosporine A inhibited the formation of ROS and delayed the onset of MPT and cell death. Addition of superoxide dismutase prevented collapse of the cytoskeleton and release of LDH from isolated hepatocytes. Ding et al. (2001) illustrated generation of superoxide and hydrogen peroxide radicals preceding microfilament disorganization and cytotoxicity. Hepatocellular glutathione levels were affected by microcystins, and administration of N-acetylcysteine was shown to protect against cytoskeletal alterations (Ding et al., 2000a).

Lipid peroxidation was observed 2 hours after exposure in the livers of male mice administered a single i.p. injection of 55 µg MC-LR/kg (purity not stated) (Wei et al., 2008). The effects of MC-LR on reactive oxygen species (ROS) and enzyme activities indicated that MC-LR-induced liver injury in mice begins with the production of ROS, which stimulated the sustained activation of JNK as well as AP-1 and Bid, changes that lead to mitochondrial dysfunction followed by apoptosis and oxidative liver injury.

Several studies have investigated the role of glutathione homeostasis and lipid peroxidation in microcystin-induced liver toxicity (Runnegar et al., 1987; Eriksson et al., 1989b; Bhattacharya et al., 1996; Ding et al., 2000a; Towner et al., 2002; Gehringer et al., 2003a,b, 2004; Bouaïcha and Maatouk, 2004). Ding et al. (2000a) indicated that microcystin exposure in isolated hepatocytes resulted in an initial increase in glutathione synthesis followed by a later depletion of glutathione. Gehringer et al. (2004) suggest that increased lipid peroxidation induced by microcystins is accompanied by an increase in glutathione peroxidase, transcriptional regulation of glutathione-S-transferase and glutathione peroxidase and *de novo* synthesis of glutathione. An intravenous LD_{50} (87 µg MC-LR equivalents/kg) of a crude microcystin extract caused a general suppression of GSTs (14 GST isoforms were measured) in both liver and testes of male rats (Li et al., 2011e). Bouaïcha and Maatouk (2004) (2 ng/mL of MC-LR in primary rat hepatocytes caused an initial increase in ROS formation and an increase in glutathione. The antioxidants vitamin E, selenium, silymarin, and glutathione provided some protection against liver toxicity and lethality from microcystins in mice (Hermansky et al. 1991; Gehringer et al., 2003a,b).

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Moreno et al. (2005) reported significant reductions in glutathione peroxidase, glutathione reductase, superoxide dismutase and catalase, along with increases in lipid peroxidation, in both the liver and kidney of rats treated intraperitoneally with single doses of MC-LR. Glutathione reductase, SOD, glutathione peroxidase, and catalase were significantly decreased while nitric oxide synthetase activity was significantly increased in both the liver and kidney of male mice administered i.p. injections of 25 µg MC-LR/kg (purified from a natural bloom of *M . aeruginosa*) every other day for a month (Sedan et al., 2010). Increases in MDA (a measure of lipid peroxidation) in the livers of mice administered crude extracts containing MC-LR (estimated dose 2.9 µg MC-LR/kg) for 21 days were reported by Li et al. (2011b). The lower doses applied in the study (0.73 and 1.5 µg/kg) did not significantly increase MDA levels. There was also no change in SOD in these animals, but there was a significant decrease in catalase.

Some studies report the absence of lipid peroxidation during microcystin-induced hepatotoxicity. In liver slices exposed to a cell extract (concentration not given), a time-dependent leakage of LDH, ALT and AST was observed with no change seen in glutathione content or lipid peroxidation by (Bhattacharya et al., 1996). In addition, Runnegar et al. (1987) suggested that glutathione depletion did not occur until after morphological changes (i.e., blebbing) were observed suggesting that ROS may not be the initiating factor for the cytoskeletal changes. This suggestion is supported by Eriksson et al. (1989b) who concluded that rapid deformation of isolated rat hepatocytes by MC-LR was not associated with alterations in glutathione homeostasis.

Lipid peroxidation was induced in the testes of immature male rabbits with a single i.p. injection of 12.5 µg MC-LR equivalents/kg of a crude extract (Liu et al., 2010). Other indicators of oxidative stress included increased hydrogen peroxide, increased catalase, SOD, glutathione peroxidase, GST and GSH.

Target Organ/Cell Type Specificity

Liver

Most oral and injection studies in laboratory animals have demonstrated that the liver is a primary target organ for microcystin toxicity. Mechanistic studies suggest that the target organ specificity is directly related to the limited ability of microcystins to cross cell membranes in the absence of an active transport system (see section 6.2.1). Liver toxicity produced by *in vitro* or *in vivo* exposures to microcystins was reduced or eliminated by inhibition of hepatocellular uptake using OATp transport inhibitors (e.g., antamanide, sulfobromophthalein and rifampicin) and bile salts (i.e., cholate and taurocholate). Lu et al. (2008) used OATp1b2 null mice to demonstrate the importance of the OATp system for transporting MC-LR into the liver.

Toxicological effects of microcystins in the isolated perfused rat liver were similar to those demonstrated following *in vivo* exposure (Pace et al., 1991). During a 60-minute exposure, MC-LR caused liver engorgement and cessation of bile flow. Electron microscopy revealed loss of sinusoidal architecture, dilation of bile canaliculi and the space of Disse and decreased intracellular contact. Mitochondrial swelling, disruption of endoplasmic reticulum and formation of whorls and loss of desmosomal intermediate filaments were also observed. Mitochondrial function was impaired, with inhibition of state 3 respiration and a decrease in the respiratory control index.

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Runnegar et al. (1995b) demonstrated cessation of bile flow, increased perfusion pressure, decreased protein secretion and decreased glucose secretion following exposure to microcystins. Histological changes included hepatocytes swelling, loss of sinusoidal architecture, pyknotic nuclei and extensive necrosis. Exposure to high concentrations of toxin extracts in the isolated perfused liver produced loss of cord architecture due to hepatocyte disassociation, membrane damage and cytolysis and nuclear effects (pyknosis, karyokinesis, karyolysis) (Berg et al., 1988). Ultrastructural effects included swollen mitochondria, vacuoles, necrosis, abnormal nuclei, bile canaliculi lacking microvilli and whorls of rough endoplasmic reticulum.

Microcystin exposure to hepatocytes in suspension or cultured in a monolayer results in membrane blebbing that becomes more pronounced and localized in one region of the cell surface. Morphological changes in hepatocytes (i.e., blebbing, rounding) have been shown to occur prior to any effect on cell membrane integrity (measured as LDH leakage or release of radiolabeled adenine nucleotides) or cell viability (generally measured as decreased trypan blue exclusion) (Runnegar et al., 1981; Runnegar and Falconer, 1982; Aune and Berg, 1986; Ding et al., 2000a).

Similar toxicological effects were observed in isolated human hepatocytes (Yea et al., 2001; Batista et al., 2003). MC-LR produced blebbing, fragmentation and hepatocyte disassociation. Cytotoxicity, as measured by LDH leakage, occurred after morphological changes were evident. Yea et al. (2001) indicated that cytotoxicity in human hepatocytes was observed at a concentration (1 μM) that did not affect rat hepatocytes. Batista et al. (2003) also reported a slightly higher susceptibility to microcystin-induced morphological change in human hepatocytes as compared to rat hepatocytes.

Thompson et al. (1988) described the time course of cellular effects of microcystins (type not specified) on cultured rat hepatocytes. Disintegration of the attachment matrix occurred by 15 minutes, followed by cells clustered in groups with no extracellular material at 1 hour, and release of cells from plates between 2 and 4 hours. LDH release did not occur until after these visual effects, and was dose-related when measured.

Inhibition of mitochondrial respiration occurred in primary hepatocytes incubated with MC-LR in the range of 0.1-50 nM (Jasionek et al., 2010). Changes in ATP levels and mitochondrial uncoupling were indicated, suggesting that MC-LR may target electron transport chain (ETC) complex I function. At noncytotoxic concentrations in HepG2 cells, microcystins interfered with the metabolism of amino acids, carbohydrates, lipids, and nucleic acids (Birungi and Li, 2011).

Kidney

An isolated perfused kidney model was used to evaluate the kidney toxicity of 1 μg MC-LR/mL (Nobre et al., 1999, 2001). MC-LR produced vascular, glomerular and tubular effects in the exposed kidney. An increase in perfusion pressure was followed by an increase in the glomerular filtration rate (GFR), increased urinary flow rate and a reduction in tubular transport at the proximal tubules. Histopathological changes included protein in the urinary spaces, but were not further described. Dexamethazone and indomethacin were shown to antagonize the effects of MC-LR, probably by blocking the MC-LR-induced activation of phospholipase A_2 and cyclooxygenase. Nobre et al. (2003) utilized rat peritoneal macrophages exposed to MC-LR to further investigate the role of inflammatory mediators in the isolated perfused kidney model. Macrophage supernatants from exposed rats caused an increase in RVR, GFR and urinary flow and reduced Na^+ transport. These effects were reduced by cyclohexamide, dexamethasone and quinacrine, further suggesting the involvement of phospholipase A_2 and other inflammatory mediators in microcystin-induced kidney toxicity.

Alverca et al. (2009) examined the effects of MC-LR (>85% pure, extracted from *M. aeruginosa* isolated from a natural bloom) on a kidney cell line (Vero-E6). Cell viability decreased in a time and dose-dependent manner. Cell morphology was affected, with enlarged lysosomes, reduction in the number of intact lysosomes, lysosomal leakage, damage to mitochondrial structure, disassembly of actin filaments, and shortening or disappearance of stress fibers observed. There was swelling of the endoplasmic reticulum cisterna and golgi apparatus vacuolization and a dose- and time related increase in apoptotic cells.

Testes

The testes are another target organ for microcystin in *in vivo* studies on male mice or rats (Li et al., 2008; Liu et al., 2010; Chen et al., 2011; Wang et al., 2012; Ding et al., 2006; Li et al., 2011b). With the exception of the Chen et al. (2011) study, dosing was by i.p. administration. The effects of a single i.p. injection of microcystin extracts from a surface bloom containing 167.7µg MC-RR/mL and 47.0 µg MC-LR/mL or 80.5 MC-LR equivalents/mL was found to have an effect on male rabbit testes. Lesions, including a variety of histological changes to both spermatogonia and Sertoli cells, were seen in animals treated with 12.5 µg MC-LR equivalents/kg; recovery occurred by 48 hours with the tissue resembling the control (Liu et al., 2010).

The *in vitro* toxicity of microcystins to Leydig cells and Sertoli cells, demonstrated by decreased cell viability (Li et al., 2008; Li & Han, 2012; Zhang et al., 2011b), suggests that microcystin uptake by the testes may be similar to that by the liver. OATps (OATP6A1) are active in the testes (Svoboda et al., 2011), although no studies to date have been located addressing their role in the testicular toxicity of microcystins. Chen et al. (2013) found that repeated i.p. dosing of rats with 10 µg MC-LR/kg affected expression of cytoskeletal genes and mitochondrial dysfunction in the testes. Levels of FSH and LH were increased; testosterone levels were decreased. Male reproductive effects were consistently observed after single and repeated parenteral exposures; these studies are described in more detail in section 7.1.2.5. Histological damage to the testes was observed in mice, rabbits, and rats administered MC-LR or a cellular extract (Chen et al., 2013; Li et al., 2008; 2011b; Liu et al., 2010; Ding et al., 2006). Sertoli cells were shown to be affected in rabbits and mice, testes and epididymal weights were decreased in mice and rats, and sperm motility and viability were affected in mice and rats.

Wang et al. (2012) evaluated the acute effects of MC-LR on gene expression and reproductive hormone levels in male BALB/c mice. Mice were injected i.p. with 0, 3.75, 7.5, 15, or 30 µg/kg of MC-LR (purity not reported) for 1, 4, 7, or 14 days. Animals in the 15 and 30 µg/kg groups lost weight over the 14 days resulting in significantly lower body weight by the end of treatment. MC-LR did not have any effect on the expression of Kisspeptin-1 (Kiss-1 which stimulates the reproductive system), GPR54 (a Kisspeptin receptor), gonadotropin releasing hormone receptor (Gnrhr), FSH receptor (Fshr), or luteinizing hormone receptor (Lhr). MC-LR caused a significant decrease of GnRH expression at all doses after 1, 4, 7, and 14 days. Fshβ was upregulated at 7.5 and 15 µg/kg, but was significantly decreased at 30 µg/kg after 14 days. Lhβ expression was significantly decreased at all doses. Changes in gene expression corresponded to increases in FSH, LH, and testosterone levels through the 7 days of treatment followed by decreases in LH and testosterone levels at all doses after 14 days of treatment. FSH levels were significantly increased at 15 µg/kg, but significantly decreased at 30 µg/kg after 14 days.

Other Tissues

Soares et al. (2007), Carvalho et al. (2010), and Casquilho et al. (2011) all observed lung damage after a single i.p. administration of MC-LR at a sublethal dose (i.e., 40 µg/kg). None of the studies detected MC-LR in the lungs, but damage was evident within 2 hours of exposure.

Lung effects include an increase in the proportion of areas with alveolar collapse accompanied by an increase in the percentage of PMN cells; increased impedance; increased oxidative stress in the lung as measured by decreased SOD, and increased catalase, TBARS, and myeloperoxidase; elevated pulmonary mechanical parameters; and increases in TNFα, IL-1β, and IL-6.

Milutinovic et al. (2006) demonstrated that 10 µg/kg of MC-LR administered i.p. every other day for 8 months to male rats caused microscopic lesions to the heart including disarray and short runs of myocardial fibers interrupted by connective tissue, increased volume density of interstitial tissue with a few lymphocyte infiltrations, enlarged cardiomyocytes with enlarged and often "bizarre-shaped" nuclei; some cells also demonstrated loss of cell cross-striations and degenerative muscle fibers with myocytolysis. A similar study by the same group using MC-YR (Šuput et al., 2010) also found similar histopathological results, but less prominent effects on the heart with MC-YR as with MC-LR. Neither MC-LR nor MC-YR induced apoptosis in the heart.

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7.1.3.5.2 Cancer Effects

Limited information from two-stage, medium-term rat liver bioassays where MC-LR was administered i.p. suggest that MC-LR can act as a promoter, increasing the number and/or size of GST (placental form)-positive foci in livers of rats pretreated with an initiating agent (Nishiwaki-Matsushima et al., 1992; Ohta et al., 1994; Sekijima et al., 1999; Hu et al., 2002). In one such study, MC-LR alone showed no initiating activity (Ohta et al., 1994). Ito et al. (1997b) observed an increase in the size of neoplastic liver nodules in mice given 100 i.p. injections of MC-LR without an initiating agent; however, the numbers of treated and control animals were small.

Studies in mice given an extract of *M. aeruginosa* in drinking water resulted in an increased mean area of aberrant colon crypt foci, although the number of foci was not affected (Humpage et al., 2000a). In other studies the total weight of skin tumors was increased in mice given an extract of *Microcystis* in drinking water after topical dimethylbenzanthracene (DMBA) pretreatment (Falconer and Buckley, 1989; Falconer, 1991). It is not possible to determine whether the observed effects resulted from exposure to microcystins or to other contaminants in the extracts.

Falconer (1991) and Falconer and Buckley (1989) reported evidence of skin tumor promotion by extracts of *Microcystis*. The extract was administered via drinking water at a concentration of 40 µg microcystin/mL to mice pretreated topically with an initiating dose of dimethylbenzanthracene (DMBA). After 52 days the total skin tumor weight in mice drinking *Microcystis* extract was significantly higher than that of mice receiving only water after initiation. The number of tumors per mouse was only slightly increased in mice receiving extract; the weight difference was largely due to the weight of individual tumors (Falconer and Buckley, 1989). The total weight of tumors in this group also exceeded that of mice pretreated with DMBA and subsequently treated with topical croton oil, with or without concurrent consumption of *Microcystis* extract. Details of the tumor incidence in the mice were not provided by the authors.

When *Microcystis* extract was provided in the drinking water (0, 10, or 40 µg/mL) of mice pretreated with two oral doses of N-methyl-N-nitroso-urea, no evidence of promotion of lymphoid or duodenal adenomas and adenocarcinomas was observed. No primary liver tumors were observed (Falconer and Humpage, 1996).

Humpage et al. (2000a) administered *M. aeruginosa* extract in drinking water to mice pretreated with azoxymethane. The content of microcystins in the drinking water was determined by mouse bioassay, HPLC, capillary electrophoresis, and protein phosphatase inhibition. The estimated doses of total microcystins were 0, 382, and 693 µg/kg/day at the midpoint of the trial. Mice were sacrificed at intervals up to 31 weeks after commencement of extract exposure. Enzyme analysis showed a concentration-dependent increase in ALP and decrease in albumin in mice treated with extract. The authors observed a concentration-dependent increase in the mean area of aberrant crypt foci of the colon, although the number of foci per colon and the number of crypts per focus were not different among the groups. Two colon tumors were found, one each in a low- and high-dose animal treated with extract. The authors proposed that increased cell proliferation caused the increase in size of foci. Histological examination of the livers of mice treated with extract showed more leukocyte infiltration in animals treated with the highest concentration of extract compared to those receiving a low concentration.

Mechanistic data indicate that at low doses, MC-LR may increase cell proliferation. MC-LR has been shown to increase the expression of the bcl-2 protein (that inhibits apoptosis) and decrease the expression of the bax protein (that induces apoptosis) (Hu et al., 2002; Lei et al., 2006; Weng et al., 2007; Li et al., 2011d). Further, MC-LR upregulates the transcription factors c-fos and c-jun, leading to abnormal proliferation (Zhao and Zhu, 2003). Gehringer (2004), in a review of the molecular mechanisms leading to promotion by MC-LR and the related tumor promoter okadaic acid, reported that MC-LR inhibits protein phosphatase PP2A, which regulates several MAPKs. The MAPK cascade regulates transcription of genes required for cell proliferation, including c-jun and c-fos. In addition, activation of the MAPK cascade has been postulated to inhibit apoptosis and thus increase cell proliferation. In addition, MC-LR has been reported to increase phosphorylation of p53 (Gehringer et al., 2004; Fu et al., 2005; Li et al., 2006; Hu et al., 2008; Xing et al., 2008; Zegura et al., 2008a; Li et al., 2011d), which is involved in regulation of the cell cycle and apoptosis.

Changes in MMP levels have been linked to cancer and tumor promotion. Zhang et al. (2010; 2012) found increased levels of MMP2 and MMP9 in the livers of male mice orally administered MC-LR for at least 180 days (subchronic and chronic results of these studies were described in Sections 7.1.2.3 and 7.1.2.6, respectively). To study further possible effects of MC-LR on tumor metastasis, Zhang et al. (2012) cultured breast cancer cells with different concentrations of MC-LR for different lengths of time. Acceleration of cell migration was found to be dependent on both the concentration and length of MC-LR incubation time. The levels of MMP2 and MMP9 were also increased with MC-LR concentration in breast cancer cells.

Birungi and Li (2011) tested the effects on noncytotoxic concentrations (1-100 ng/mL) of MC-LR, MC-YR, and MC-RR on HepG2 cells. While higher concentrations (1000 ng/mL) are known to cause cell death, cells continued to proliferate at the noncytotoxic concentrations used in this study. The study authors suggested this could lead to uncontrolled growth and possibly

tumors. MC-LR (10 µg/L) incubated with WRL-68 cells, a human cell line, for 25 passages had an increase growth rate compared to controls (Xu et al., 2012). Gan et al. (2010b) also found that MC-LR enhanced cell proliferation in the liver cancer cell lines HepG2 and Hep3B. MC-LR was also found to activate nuclear factor erythroid-2 (Nrf2) in a dose-dependent manner. Inhibiting Nrf2 also inhibited MC-LR-induced cell proliferation.

Nong et al. (2007) incubated HepG2 cells with 100 µM MC-LR for 24 or 48 hours. After both time periods there was an increase in the number of cells in G0/G1 phase of the cell cycle with less in the S phase of the cell cycle. ROS scavengers (catalase, SOD, or deferoxamine) did not affect the blockage in the cell cycle induced by MC-LR. The opposite was observed in a kidney cell line.

Dias et al. (2010) studied the effects of MC-LR on the proliferation of nonhepatic cells using a kidney epithelial cell line (Vero-E6). Previous studies (Dias et al., 2009; Alverca et al., 2009) had found MC-LR cytotoxic to this cell line, at doses as low as 11 µM. Therefore, Dias et al. (2010) used commercial MC-LR (purity ≥95%) or extracted MC-LR (purity not reported, but stated to have been tested) in the range of 5-5000 nM. Even the lowest concentration caused an increase in ERK1/2 activity, suggesting that MC-LR stimulates the G1/S transition and activates the ERK1/2 pathway (as noted by increases in p38, JNK, and ERK1/2 activity) in kidney cells.

7.1.4 Structure-Activity Relationships

With a few exceptions, microcystin congeners exhibit i.p. LD_{50} values between 50 and 300 µg/kg in mice (Rinehart et al., 1994; WHO, 1999). MC-LR is one of the most potent congeners (i.p. LD_{50} approximately 50 µg/kg). Limited comparative testing of *in vitro* protein phosphatase inhibition (IC_{50}) of MC-LR, -RR and -YR resulted in IC_{50} values of 1.6, 3.4 and 1.4 nM, respectively (Yoshizawa et al., 1990), indicating that microcystin congeners may be relatively similar in protein inhibition potency despite differences in the variable amino acids. Pharmacokinetic differences among the various microcystin congeners may be at least partially responsible for observed variations in lethal potency (Ito et al., 2002b). Microcystin congeners of varying hydrophobicity were shown to interact differently with lipid monolayers (Vesterkvist and Meriluoto, 2003). Effects on membrane fluidity could alter the cellular uptake of these toxins.

Wolf and Frank (2002) proposed toxicity equivalency factors (TEFs) for the four major microcystin congeners based on LD_{50} values obtained after i.p. administration. The proposed TEFs, using MC-LR as the index compound (TEF=1.0) were 1.0 for MC-LA and MC-YR and 0.1 for MC-RR. The application of TEFs based on i.p. LD_{50} values to assessment of risk from oral or dermal exposure is questionable given that differences in liphophilicity and polarity of the congeners may lead to variable absorption by non-injection routes of exposure. However, the differences between the LD_{50} information and the IC_{50} data reduce confidence in the LD_{50} derived TEFs.

Commented [IS53]: Why do differences in LD50s and IC50s from *in vitro* studies diminish the reliability of *in vivo* investigations? Why not the other way round? Aren't these studies (*in vitro* and *in vivo*) asking different questions and therefore not directly comparable?

7.1.5 Hazard Characterization

7.1.5.1 Synthesis and Evaluation of Major Noncancer Effects

The preponderance of toxicological data on the effects of microcystins is restricted to the MC-LR congener. A single, poorly-described study, reported only in a secondary source is available for the LA congener. Data on the YR and RR congeners are limited to i.p. LD_{50} values and measures of relative inhibition of protein phosphatases; in addition, MC-YR has been shown to exert greater cytotoxicity than MC-RR (Puerto et al., 2009). As a result, this section largely describes the available information on the toxic effects of MC-LR, with limited reference to other congeners.

Commented [IS54]: The single, poorly-described study should be cited at the end of this sentence

Anecdotal reports and observational studies indicate that, in humans, exposure to cyanobacterial blooms (including microcystin-producing genera) can result in neurological, gastrointestinal and dermatological symptoms, such as headache; muscle weakness; eye, ear and throat irritation; nausea; stomach pain; diarrhea; blistering around the mouth; and hay-fever like symptoms (Dillenberg and Dehnel, 1960; Billings, 1981; Turner et al., 1990; Teixeira et al., 1993; el Saadi and Cameron, 1993). Effects were reported in persons exposed via recreational contact (swimming, boating) and drinking water. Turner et al. (1990) also reported pneumonia in army recruits exposed to a cyanobacterial bloom. Symptoms occurring after exposure to cyanobacteria cannot be directly attributed to microcystin toxins (or endotoxins); some effects may result from exposure to the cyanobacterial cells themselves, or from exposure to multiple toxins in the bloom.

Commented [IS55]: I'd argue that symptoms like headache and muscle weakness are not strictly neurological in the context of anecdotal and case reports, and epidemiological studies of exposure to microcystin-producing cyanobacteria in recreational waterbodies. They're likely part of the broader sweep of flu-like symptoms reported after such exposures

A major noncancer health effect of exposure to MC-LR is liver damage. Severe liver damage (diffuse individual hepatocyte necrosis, cell-plate disruption and apoptosis) occurred in dialysis patients exposed to microcystins via untreated water containing cyanobacteria in the dialysate (Jochimsen et al., 1998; Pouria et al., 1998; Carmichael et al., 2001; Azevedo et al., 2002). At high acute doses in laboratory animals, MC-LR caused potentially fatal hemorrhaging within the liver. While the liver is the usual target of microcystin toxicity, there have been some reports of effects in other systems, including hematological, kidney, cardiac, neurological, reproductive, and gastrointestinal effects. It has been suggested that some effects in other organs observed after high doses of MC-LR may result from ischemia or hypoxia caused by hepatic hemorrhage. However, some effects outside the liver have been observed in the absence of hemorrhage. Notably, evidence for effects of MC-LR on the male reproductive system and sperm development has been found. Effects on testicular cells have been observed following both *in vivo* and *in vitro* exposures, suggesting that this effect is not secondary to liver toxicity (Chen et al., 2011; Li et al., 2011b; Liu et al., 2010; Kirpenko et al., 1981; Li et al., 2008; Zhang et al., 2011b).

Much of the toxicological data on microcystins are limited to reports of liver effects after single lethal or sublethal doses administered via i.p. injection. These studies indicate that injected doses of 50-200 µg/kg of MC-LR or MC-YR are usually lethal in mice and rats within a few hours (Adams et al., 1988; Hooser et al., 1989a; Hermansky et al., 1990c; Stotts et al., 1993; Gupta et al., 2003; Rao et al., 2005). Miura et al. (1991) showed that the median time to death is greatly increased in fed rats (32 hours) when compared with fasted rats (less than 2 hours). The authors suggest that fasting may increase the sensitivity of animals to the mitochondrial toxicity of microcystins, although this could not be conclusively demonstrated. In the liver, MC-LR destroys the cytoskeleton of hepatocytes, leading to hepatocyte disassociation, degeneration,

apoptosis and necrosis (Hermansky et al., 1990c; Hooser et al., 1991b). Hepatic hemorrhage and disintegration of the liver architecture follow quickly (Hooser et al., 1991b).

Effects reported to occur outside the liver include pulmonary thrombi derived from necrotic hepatocytes, kidney effects such as dilation of cortical tubules and eosinophilic material in the cortical tubules, and degeneration and necrosis of myocardial cells (Adams et al., 1988; LeClaire et al., 1988; Zhang et al., 2002). As previously stated, some of these effects may occur secondarily to hepatic hemorrhage.

Effects have been observed in the male reproductive system including decreased absolute and relative testes weight; decreased absolute and relative epididymis weight; decreased epididymidal sperm concentration, sperm viability, and proportion of sperm with rapid progressive motility; increased percent immobile sperm and sperm abnormality; atrophy of the seminiferous tubules with greater spaces between the seminiferous tubules, obstructed seminiferous tubules, deformation of androgonial and sperm mother cells; decreased number of interstitial cells, Sertoli cells, and mature sperm in the seminiferous tubule; lipid peroxidation; and apoptosis (Chen et al., 2011; Li et al., 2011b; Liu et al., 2010; Kirpenko et al., 1981; Li et al., 2008; Zhang et al., 2011b).

Commented [IS56]: This single sentence should be broken up…

Injection studies suggest a very steep dose-response curve for acute liver effects from microcystin exposure. In several studies, mice and rats receiving single i.p. doses of 20-40 µg/kg MC-LR showed no clinical toxicity and few or no gross or microscopic effects in the liver or other organs (Hooser et al., 1989a; Lovell et al., 1989; Hermansky et al., 1990c), while i.p. doses of 50-200 µg/kg are usually lethal within a few hours (Hooser et al., 1989a; Hermansky et al., 1990c; Stotts et al., 1993; Gupta et al., 2003).

7.1.5.2 Oral

Table 7-14 provides a summary of the noncancer effects from oral studies of MC-LR or microcystin extract toxicity in laboratory animals. As the table indicates, the toxicological database for effects of microcystins after oral exposure is limited.

Liver Effects

One study of human exposure to drinking water before, during and after a bloom of *M. aeruginosa* reported a significant increase in GGT levels during the bloom compared with levels before the bloom as well as compared to the levels in patients living in areas served by other water supplies (Falconer et al., 1983). The study population consisted of all persons subjected to liver function tests in the area served by the affected drinking water supply; as such, it is not representative of the general population. Another study evaluated liver damage in children in relation to the microcystin levels in drinking water and aquatic food (carp and duck) in China (Li et al., 2011a). Microcystin levels were associated with increasing levels of AST and ALP, but not ALT and GGT. The OR for liver damage (increased serum enzyme levels) in exposed children was 1.72 (95% CI: 1. (?)05-2.76). The liver is a target organ when laboratory animals are exposed to high doses of MC-LR. Oral exposure to single 500 µg/kg doses of MC-LR caused diffuse hemorrhage in the liver of mice and rats; more pronounced liver damage occurred

at higher doses (Ito et al., 1997a; Fawell et al., 1999a). Young mice (5 weeks old) did not develop signs of hepatotoxicity at 500 μg/kg of MC-LR, while aged mice (32 weeks old) developed clear signs (Ito et al., 1997a). This difference may result in part from variable gastrointestinal

Table 7- 14. Summary of Noncancer Results in All Animal Studies of Oral Exposure to Microcystins

Species	Sex	Average Daily Dose (µg/kg-day)	Exposure	NOAEL (µg/kg-day)	LOAEL (µg/kg-day)	Responses	Comments	Reference
Acute Exposure								
Rat	M/F	500, 1580, 5000	Single gavage	ND	500	Mortality; diffuse hepatic hemorrhage at all doses	No untreated controls. Dose-dependent increase in hepatotoxicity	Fawell et al., 1999a
Mouse	M/F	500, 1580, 5000	Single gavage	ND	500	Mortality; diffuse hepatic hemorrhage at all dose	No untreated controls. Dose-dependent increase in hepatotoxicity	Fawell et al., 1999a
Mouse	F	8000, 10000, 12500	Single gavage	ND	8000	Hypertrophic hepatocytes, fibrosis in centrilobular and midzonal regions; mortality (2/2) at highest dose	No untreated controls. 1-2 animals/dose group.	Yoshida et al., 1997
Mouse	M	0, 500	Single gavage	ND	500 (aged mice only)	Centrilobular hepatic hemorrhage and necrosis; necrosis of intestinal mucosa and duodenal damage	Effects observed in aged (32 week-old) mice; no effects on liver or gastrointestinal tract in young (5 week-old) mice	Ito et al., 1997a
Short-Term Exposure								
Rat	M	0, 50, 150	Drinking water, 28 day	ND	50	Slight to moderate degenerative and necrotic hepatocytes with hemorrhages; increased serum enzymes (ALP and LDH)		Heinze,1999
Mouse	M	0, 4.6, 23, 46, 93, 186	Gavage, 7 days	4.6	23	Dose-related increase in apoptotic cells in liver; increased Bax expression; decreased Bcl-2.		Huang et al. 2011
Mouse	M/F	43.3 - 333.3	Dietary; duration not given	ND	ND	No effects on clinical signs, body weight, or mortality	*A. flos-aquae* as food supplement; MC-LR content estimated	Schaeffer et al., 1999

Species	Sex	Average Daily Dose (µg/kg-day)	Exposure	NOAEL (µg/kg-day)	LOAEL (µg/kg-day)	Responses	Comments	Reference
Subchronic Exposure								
Mouse	M/F	0, 40, 200, 1000	Daily gavage, 13 weeks	40	200	Minimal/slight chronic inflammation with hemosiderin deposits and single hepatocyte degeneration; increased serum enzymes (ALT and AST)		Fawell et al., 1999a
Pig	Not given	Not reported	Drinking water; 44 days	ND	ND	Increases in GGT, ALP, total bilirubin; decreased albumin; liver histopath.	80-374 mg dried algae/kg added to drinking water	Falconer et al., 1994
Chronic Exposure								
Mouse	F	0, 3	Drinking water, 18 months	3	ND	No effects on survival, body weight, hematology, serum biochemistry, organs or histopathology	Minor changes in ALP and cholesterol not considered toxicologically significant	Ueno et al., 1999
Mouse	Not given	Not available	Gavage, 80 µg/kg, 80-100 times over 28 weeks	ND	80	Light injuries to hepatocytes in the vicinity of the central vein	Only liver examined; only three control animals; dosing frequency unclear	Ito et al., 1997b
Mouse	M	0, 0.2, 8.0, 16	Drinking water, 180 or 270 days	0.2	8	Histopathological changes in the liver, decreased body weight, increased relative liver weight; MMP expression increased in all groups	Changes in MMP expression and protein levels not considered adverse.	Zhang et al., 2010; 2012
Developmental Toxicity								
Mouse	M	0, 0.25, 0.79, 2.5 (calculated)	Drinking water; 3 or 6 months	0.25	0.79	Decreased sperm counts and motility; lesions in the testes; decreased testosterone, increased LH and FSH		Chen et al., 2011

Species	Sex	Average Daily Dose (µg/kg-day)	Exposure	NOAEL (µg/kg-day)	LOAEL (µg/kg-day)	Responses	Comments	Reference
Rat	M/F	0.0005 or 0.5 (extract) 10 mg/kg (biomass)	Oral; 3 months	0.0005	0.5	Histopath lesions in ovaries and testes; changes in estrous cycle; multiple sperm effects	5×10^{-4} or 5×10^{-7} mg/kg extract; or 10 mg/kg biomass	Kirpenko et al., 1981
Mouse	F	0, 200, 600, 2000	Gavage, GD 6-15	600	2000	Maternal mortality (7/26) and morbidity (2/26 humanely sacrificed); reduced fetal body weight, delayed skeletal ossification	Data on reproductive or developmental parameters were not included.	Fawell et al., 1999a

absorption of microcystins, but cannot be entirely explained by absorption differences, since similar age-dependent effects were reported after i.p. exposure (Adams et al., 1985; Rao et al., 2005).

A single 28-day study of oral exposure to 50 or 150 µg/kg of MC-LR in drinking water showed increased liver weight, slight to moderate liver lesions with hemorrhages and increased ALP and LDH in rats exposed at 50 µg/kg-day (Heinze, 1999). A subchronic study in mice using a similar dose range identified a LOAEL of 200 µg/kg (Fawell et al., 1999a). At this dose, mild liver lesions including chronic inflammation, hemosiderin deposits and single hepatocyte degeneration were observed, as well as increased ALT and AST in male animals. No effects were identified at a dose of 40 µg/kg. Mild hepatocyte injury was reported in mice given 80 or 100 gavage doses of 80 µg/kg each over 28 weeks, corresponding to time-weighted average doses of 33-41 µg/kg-day (Ito et al., 1997b). Based on the report, it appears that a limited postmortem examination was conducted in this study, which was primarily aimed at evaluating carcinogenicity. No liver or other toxicity was reported in female mice given approximately 3 µg/kg-day MC-LR in drinking water for 18 months (Ueno et al., 1999). Male mice administered 8 or 16 µg MC-LR/kg/day via the drinking water for 180 days showed mild hepatocyte injury and increased relative liver weight (accompanied by decreased body weight) (Zhang et al., 2010).

Testes Effects

MC-LR has been observed to affect the testes at low doses following oral exposure. Male mice administered MC-LR via their drinking water for 3 or 6 months at low concentrations (3.2 µg/L equivalents to 0.79µg/kg/day) had decreased sperm counts and sperm motility (Chen et al., 2011). By 6 months there was also an increase in sperm abnormalities, decreased serum testosterone and increased serum LH levels. Testes weights, however, were not affected. Similar adverse effects on sperm were observed in rats administered 0.5 µg/kg of toxin extract or 10 mg/kg of biomass of *M. aeruginosa* for three months (Kirpenko et al., 1981).

Effects on the male reproductive system have also been observed with pure MC-LR and extracts in *in vivo* studies using parenteral administration (Ding et al., 2006; Li et al., 2008; Li et al., 2011b) and in *in vitro* studies (Li et al., 2008; Zhang et al., 2011b; Li and Han, 2012). *In vivo* changes on sperm include decreased sperm motility and viability, sperm counts, spermatogonia and spermatid quality; and increase in abnormal sperm (Ding et al., 2006; Li et al., 2008). Numerous histological changes have also been observed in the testes including testicular atrophy and degeneration; depopulation of the Leydig, Sertoli, and mature sperm cells; and increased apoptosis (Ding et al., 2006; Li et al., 2008). Changes in testes weight have been variable and possibly related to changes in body weight (Ding et al., 2006), but absolute and relative epididymis weight has been found to be decreased (Ding et al., 2006). *In vitro* cell viability of Sertoli and Leydig cells was decreased by exposure to MC-LR (Li et al., 2008; Zhang et al., 2011b; Li and Han, 2012). Changes in morphology were marked by cell shrinkage and loss of membrane integrity. Wang et al. (2012), however, observed that MC-LR was not able to enter Leydig cells and found no MC-LR induced cytotoxicity in the Leydig cells. Lipid peroxidation and apoptosis has also been observed *in vitro* with MC-LR exposure to Sertoli and Leydig cells (Liu et al., 2010; Zhang et al., 2011b). Male hormone levels have been found to be

effected in both *in vitro* and *in vivo* studies. *In vivo* studies in male mice found MC-LR induced decreases in serum testosterone and increases in LH and FSH serum (Li et al., 2008). Testosterone production was also decreased *in vitro* in Leydig cells incubated with MC-LR (Li et al., 2008).

Neurological Effects

The literature contains scattered reports of neurological symptoms after exposure to high doses of MC-LR. Dialysis patients exposed to microcystins (and cylindrospermopsin) in dialysate reported symptoms such as visual disturbance, blindness, vertigo, headache and muscle weakness (Jochimsen et al., 1998). Clinical signs in mice and rats orally exposed to lethal doses (about 5000 µg/kg) include hypoactivity and piloerection (Fawell et al., 1999a). Direct injection of MC-LR into the (mouse) brain (1 or 10 µg/L) was associated with impaired memory function (Li et al., 2012).

Commented [IS57]: Hypoactivity and piloerection in rats are arguably not neurological signs, but components of non-specific sickness behavior. Likewise the signs and symptoms from the Caruaru incident (visual disturbance, vertigo, headache, muscle weakness) may not be, strictly speaking, neurological in origin, but part of the bigger picture of a severe acute hepatic syndrome. The direct injection of MC-LR and reported memory dysfunction would seem to meet the criteria for description as neurological signs, however.

Other Organs

Gastrointestinal effects (necrosis, duodenal damage) were observed in aged mice exposed orally to single 500 µg/kg doses of MC-LR (Ito et al., 1997a). Kidney effects including eosinophilic materials in the Bowman's spaces were observed in two mice exposed to a lethal dose of 12.5 mg/kg (Yoshida et al., 1997). Female mice exposed subchronically to 1000 µg/kg had slight increases in hemoglobin concentration, erythrocyte count and packed cell volume (Fawell et al., 1999a). Milutinovic et al. (2002, 2003) briefly reported that kidney effects are more pronounced than liver effects in rats chronically exposed to i.p. doses of MC-LR and MC-YR (time weighted average dose, 5 µg/kg for 8 months). Details of the liver examinations were not reported in this study, limiting the usefulness of these data.

Developmental Effects

A single oral study of developmental toxicity in mice reported maternal toxicity, liver effects and deaths in some dams treated at the highest dose of MC-LR (2000 µg/kg during GD 6-15), along with reduced fetal body weight and delayed skeletal ossification. No effects on reproductive or developmental parameters were observed in other treatment groups, and 600µg/kg was identified as a NOAEL for developmental toxicity (Fawell et al., 1999a). One study of developmental toxicity after i.p. injection of 32-238 µg MC-LR/kg in mice confirmed the lack of developmental or reproductive effects in the absence of maternal toxicity (Chernoff et al., 2002). A study in which an extract of *M. aeruginosa* (estimated to contain about 14 µg/L unspecified toxin) was administered in the drinking water to mice before and during pregnancy revealed small brains in 7 of 73 pups from treated parents and none in untreated controls (Falconer et al., 1988). The litter distribution of the affected pups was not reported by the authors. It is not possible to attribute this effect to microcystin exposure, as the extract may have contained other compounds.

In vitro studies suggest that MC-LR can disrupt the cytoskeleton of embryonic cells, causing cell detachment, retarding division or causing cytolysis (Sepulveda et al., 1992; Frangez et al., 2003; Zuzek et al., 2003). MC-LR effects on these and other cell types may be limited by

the degree of uptake. Frangez et al. (2003) showed that an intact zona pellucida prevented effects in rabbit whole embryo cultures.

7.1.5.3 Inhalation

Very limited information is available on the toxicity of MC-LR via inhalation exposure. The available data indicate that short-term inhalation of a low concentration of MC-LR can cause local damage to the epithelial cells of the nasal cavity. A single study of inhalation exposure in mice revealed dose-dependent damage to the respiratory and olfactory epithelial cells of the nasal cavity (Benson et al., 2005). Exposure occurred over 7 days at 260 $\mu g/m^3$ for 30, 60 and 120 minutes per day (authors estimated the deposited doses as 3, 6 and 12 μg/kg). No effects on the liver or other organs were observed.

Several limited lines of evidence suggest that high doses of MC-LR via respiratory exposure routes can lead to systemic uptake with subsequent liver effects. Systemic uptake of MC-LR by respiratory routes of exposure has been demonstrated in studies of acute, high-dose exposure (Creasia, 1990; Fitzgeorge et al., 1994; Ito et al., 2001). Importantly, the LD_{50} for MC-LR given via either intranasal or intratracheal instillation is similar to that of MC-LR given via i.p. injection (Fitzgeorge et al., 1994; Ito et al., 2001). As with i.p. and oral exposure, liver hemorrhage is the proximate cause of death in animals lethally dosed via intranasal or intratracheal instillation (Fitzgeorge et al., 1994; Ito et al., 2001). Further evidence of systemic effects comes from a brief abstract describing lethality in mice exposed via inhalation (nose only) to MC-LR aerosols. Creasia (1990) reported an LC_{50} for MC-LR of 18 mg/m^3 air for 10 minutes (authors estimated the deposited dose as 45 μg/kg), and indicated that histopathological findings in deceased mice were similar to those reported after i.v. dosing. Ito et al. (2001) suggested that MC-LR could enter the bloodstream either via local damage to the nasal mucosa leading to exposure of the nasal blood vessels, or through transport to the lung and absorption into alveolar capillaries.

7.1.5.4 Synthesis and Evaluation of Carcinogenic Effects

Several human epidemiological studies have reported an association between consumption of drinking water containing cyanobacteria and microcystins and liver or colon cancer in certain areas of China (Yu et al., 1989 and Yu, 1989 as cited in Ueno et al., 1996; Zhou et al., 2002). In all of these studies, the use of a surface drinking water supply was used as a surrogate for exposure to microcystins. Individual exposure to microcystins was not estimated. Further, it is not clear whether these studies adequately controlled for confounding factors, such as hepatitis infection or aflatoxin exposure in drinking water.

Ito et al. (1997b) conducted the only longer-term oral study of a purified microcystin. In this study, chronic gavage doses of 80 μg MC-LR/kg/day over 28 weeks failed to induce neoplastic nodules of the liver in mice; but these data are of limited value for cancer assessment because of the short exposure duration. As part of the same study, Ito et al. (1997b) found an increase in the size of neoplastic liver nodules in mice given 100 i.p. injections of MC-LR without an initiating agent; however, the numbers of treated and control animals were small. Limited information from two-stage, medium-term rat liver bioassays where MC-LR was

administered i.p. suggest that MC-LR can act as a promoter, increasing the number and/or size of GST-P positive foci in livers of rats pretreated with an initiating agent (Nishiwaki-Matsushima et al., 1992; Ohta et al., 1994; Sekijima et al., 1999; Hu et al., 2002). In one such study, MC-LR alone showed no initiating activity (Ohta et al., 1994).

7.1.5.5 Mode of Action and Implications in Cancer Assessment

A number of studies provide support for identification of microcystins as tumor promoters rather than initiators. In mice given an extract of *M. aeruginosa* in drinking water, the mean area of aberrant crypt foci of the colon was significantly increased, although the number of foci was not affected (Humpage et al., 2000a). Similarly, the total weight of skin tumors was increased in mice given an extract of *Microcystis* in drinking water after topical DMBA pretreatment (Falconer and Buckley, 1989; Falconer, 1991). It is not possible to determine whether the observed effects resulted from exposure to microcystins or to other contaminants in the extracts.

Mechanistic data indicate that at low doses, MC-LR may increase cell proliferation. MC-LR has been shown to increase the expression of the bcl-2 protein (that inhibits apoptosis) and decrease the expression of the bax protein (that induces apoptosis) (Hu et al., 2002; Lei et al., 2006; Weng et al., 2007; Li et al., 2011d). Further, MC-LR upregulates the transcription factors c-fos and c-jun, leading to abnormal proliferation (Zhao and Zhu, 2003). Gehringer (2004), in a review of the molecular mechanisms leading to promotion by MC-LR and the related tumor promoter okadaic acid, reported that MC-LR inhibits protein phosphatase PP2A, which regulates several MAPKs. MC-LR has been observed to increase MAPK phosphorylation in livers (Zhang et al., 2010). The MAPK cascade regulates transcription of genes required for cell proliferation, including c-jun and c-fos. In addition, activation of the MAPK cascade has been postulated to inhibit apoptosis and thus increase cell proliferation. Finally, MC-LR has been reported to increase phosphorylation of p53 (Gehringer et al., 2004; Fu et al., 2005; Li et al., 2006; Hu et al., 2008; Xing et al., 2008; Zegura et al., 2008a; Li et al., 2011d), which is involved in the regulation of the cell cycle and apoptosis.

Changes in MMP levels have been linked to cancer and tumor promotion. Zhang et al. (2010; 2012) found increased levels of MMP2 and MMP9 in the livers of male mice orally administered MC-LR for at least 180 days (subchronic and chronic results of these studies were described in Sections 7.1.2.3 and 7.1.2.6, respectively). To study further possible effects of MC-LR on tumor metastasis, Zhang et al. (2012) cultured breast cancer cells with different concentrations of MC-LR for different lengths of time. Acceleration of cell migration was found to be dependent on both the concentration and length of MC-LR incubation time. The levels of MMP2 and MMP9 were also increased with MC-LR concentration in the breast cancer cells.

Birungi and Li (2011) tested the effects on noncytotoxic concentrations (1-100 ng/mL) of MC-LR, MC-YR, and MC-RR on HepG2 cells. While higher concentrations (1000 ng/mL) cause cell death, cells continued to proliferate at these noncytotoxic concentrations. The study authors suggested this could lead to uncontrolled growth and possibly tumors. MC-LR (10 µg/L) incubated with WRL-68 cells, a human cell line, for 25 passages had an increase growth rate compared to controls (Xu et al., 2012). Gan et al. (2010b) also found that MC-LR enhanced the cell proliferation in the liver cancer cell lines HepG2 and Hep3B. MC-LR was also found to

activate nuclear factor erythroid-2 (Nrf2) in a dose-dependent manner. Inhibiting the Nrf2 also inhibited the MC-LR-induced cell proliferation. Nong et al. (2007) incubated HepG2 cells with 100 μM MC-LR for 24 or 48 hours. After both time periods there was an increase in the number of cells in G0/G1 phase of the cell cycle with less in the S phase of the cell cycle. ROS scavengers (catalase, SOD, or deferoxamine) did not affect the blockage in the cell cycle induced by MC-LR. The opposite was observed in a kidney cell line. Dias et al. (2010) studied the effects of MC-LR on the proliferation of nonhepatic cells using a kidney epithelial cell line (Vero-E6). Previous studies (Dias et al., 2009; Alverca et al., 2009) had found MC-LR cytotoxic to this cell line, at doses as low as 11 μM. Therefore, Dias et al. (2010) used commercial MC-LR (purity ≥95%) or extracted MC-LR (purity not reported, but stated to have been tested) in the range of 5-5000 nM. Even the lowest concentration caused an increase in ERK1/2 activity, suggesting that MC-LR stimulates the G1/S transition and activates the ERK1/2 pathway (as noted by increases in p38, JNK, and ERK1/2 activity) in kidney cells.

Genotoxicity studies of MC-LR have given conflicting results. Two microcystin-containing extracts induced mutations in the Ames assay (Ding et al., 1999; Huang et al., 2007), while negative results have also been observed using *M. aeruginosa* extracts, as well as purified microcystin (Grabow et al., 1982; Wu et al., 2006; Repavich et al., 1990). Using an umuC assay in *Salmonella typhimurium* TA 1535/pSK1002, MC-LR had genotoxic activity (Sieroslawska and Rymuszka, 2010). Positive results have been observed with mammalian cell lines (Suzuki et al., 1998; Zhan et al., 2004; Nong et al., 2007; Zegura et al., 2006, 2008a,b, 2011; Li et al., 2011b), but *in vivo* animal studies have conflicting results (Gaudin et al., 2008, 2009; Abramsson-Zetterberg et al., 2010; Zhang et al., 2011a; Dong et al., 2008). Evidence for MC-LR-induced DNA damage as measured by the comet assay has been called into question by the finding that apoptosis can lead to false positive findings in this assay (Lankoff et al., 2004a). Some evidence exists for a clastogenic effect of MC-LR (Ding et al., 1999; Zhan et al., 2004; Lankoff et al., 2006a; Repavich et al., 1990). Metabolic activation has been found to decrease MC-LR mutagenicity.

The inconsistent information on mutagenicity may be related to differences in the cell uptake of MC-LR or the metabolism of MC-LR in the test system. DNA fragmentation was significantly increased in rat neutrophils with MC-LA and MC-YR, but not in human neutrophils (Kujbida et al., 2008). MC-YR has also been found to induce DNA damage in the blood (lymphocytes), liver, kidney, lung, spleen, and brain of mice administered 10 μg MC-YR/kg via i.p. injection every other day for 30 days (Filipič et al., 2007). Lankoff et al. (2003) showed that MC-LR, through its effect on microtubules, damages the mitotic spindle, leading to the formation of polyploid cells.

7.1.5.6 Weight of Evidence Evaluation for Carcinogenicity

Applying the Guidelines for Carcinogen Risk Assessment (U.S. EPA, 2005a), there is *inadequate evidence to determine the carcinogenicity* of microcystins. The few available epidemiological studies that suggest a positive association between liver or colorectal cancers and microcystin exposures are limited by ecological study design, poor measures of exposure, potential co-exposure to other microbial or chemical contaminants and, in some cases, failure to control for known liver and colorectal risk factors. No long term animal studies designed to evaluate dose-response of the tumorigenicity of microcystin following lifetime exposures were

available. MC-LR has been shown to have a promotional effect in two-stage rat liver bioassays using i.p. administration; however, the relevance of this effect to environmental exposures is uncertain. Mechanistic information provides some support for a possible promotional effect of MC-LR.

7.1.5.7 Potentially Sensitive Populations

Little information is available on potentially susceptible populations. Studies in laboratory rodents suggest that the acute effects of MC-LR may be more pronounced in adult or aged animals than in juvenile animals (Adams et al., 1985; Ito et al., 1997a; Rao et al., 2005). In these studies, young animals showed little or no effect at MC-LR doses lethal to adult animals. Age-dependent differences in toxicity were observed after both oral and i.p. exposure, suggesting that differences in gastrointestinal uptake were not entirely responsible for the effect of age. The relevance of these age-related differences to acute toxicity in humans is unclear.

Available information does not suggest any pronounced gender differences in response to microcystins. Studies with algal extracts suggest the possibility that male mice may be more sensitive than female mice to oral exposure to algal extracts (Falconer et al., 1988). However, the relevance of this finding to human microcystin exposure is uncertain given the potential for co-exposure to other constituents and toxins in algal extracts.

Because microcystins inhibit the action of protein phosphatases (PP1 and PP2A), co-exposure to other compounds that inhibit these enzymes may enhance the toxicological effects of microcystins.

7.2 Anatoxin-a

7.2.1 Animal Studies

7.2.1.1 Acute Toxicity

Oral Exposure

An acute oral (single dose gavage) LD_{50} value of 16.2 mg/kg (95% confidence interval [CI]: 15.4-17.0) was determined for synthetic (+)-anatoxin-a hydrochloride (commercial product, > 98% pure) in adult male Swiss Webster ND-4 mice (Stevens and Krieger, 1991b). LD_{50} values were determined using the method of moving averages for four doses with six animals per dose. The 16.2 mg/kg LD_{50} is equivalent to 13.3 mg anatoxin-a/kg (95% CI: 12.8-14.1). In the same study, a single dose gavage LD_{50} value of 6.7 mg/kg (95% confidence interval [CI]: 6.3-7.1) was determined for adult male Swiss Webster ND-4 mice administered the toxin as a lysate solution of lyophilized *A. flos-aquae* (NRC-44-1) cells (Stevens and Krieger, 1991b).

A single dose gavage LD_{50} of >5 mg/kg was determined for anatoxin-a in groups of 6 newly weaned CBA/BalbC mice of unspecified sex (Fitzgeorge et al., 1994); the anatoxin-a in this study was a commercial product in a "suitably purified" but unspecified form. Deaths

occurred within 2 minutes of gavage administration and were due to neurotoxicity, with manifestations that included loss of coordination, muscular twitching and death by respiratory paralysis (Fitzgeorge et al., 1994).

Anatoxin-a has been implicated in case reports of poisonings and deaths in dogs, livestock, and waterfowl that consumed water containing blooms of toxin-producing cyanobacteria (Carmichael and Gorham, 1978; Edwards et al., 1992; Gunn et al., 1992; Pybus et al., 1986). Signs of toxicity were predominantly neurologic, with deaths due to respiratory paralysis. Quantitative exposure data for cyanotoxins were not reported.

A 5-day oral toxicity study was performed in which groups of two male and two female Crl:CD-1(ICR)BR mice were administered aqueous (+)-anatoxin-a hydrochloride (commercial product, purity not reported) by gavage in daily doses of 1.5, 3, 7.5 or 15 mg/kg/day (equivalent to 1.2, 2.5, 6.2 or 12.3 mg anatoxin-a/kg/day) (Fawell and James, 1994; Fawell et al., 1999b). The study was conducted to determine the maximum tolerated dose to be used in the 28-day study summarized below. The dosing of the 6.2 and 12.3 mg/kg-day groups commenced approximately 24 hours after the dosing of the 1.2 mg/kg-day group. The 2.5 mg/kg-day group was established 5 days after dosing of the 6.2 and 12.3 mg/kg-day groups as an intermediate level due to toxicity at these dosages (discussed below). No control group was included. Clinical signs, body weight and food consumption were assessed, and surviving animals were necropsied. All high-dose mice and one female mouse in the 6.2 mg/kg-day group died within 5 minutes of dosing. Males in the 6.2 mg/kg-day dose group were hyperactive following the third dose; no other signs of neurotoxicity were reported and none of the other surviving animals had any abnormal clinical signs. No changes in body weight or food consumption or unusual necropsy findings were observed in any animals. The highest NOAEL, 2.5 mg/kg-day (3 mg anatoxin-a hydrochloride/kg-day), was identified by the authors as the maximum tolerated dose for the 28-day main study.

Other Exposure Routes

Acute (single dose) LD_{50} values have been determined in mice following i.p. exposures; values include 0.25 mg/kg (95% CI: 0.24-0.28) for (+)-anatoxin-a hydrochloride (commercial product, >98% pure) (0.21 mg anatoxin-a/kg) (Stevens and Krieger, 1991b) and 0.375 mg/kg for commercial anatoxin-a (form and purity not reported) (Fitzgeorge et al., 1994). Lethal i.p. doses were characterized by neurotoxic effects that included loss of coordination, muscular twitching and death by respiratory failure within 2 minutes (Fitzgeorge et al., 1994).

Valentine et al. (1991) compared acute lethality in male BalbC mice that were administered single i.p. injections of (+)-, racemic or (-)-anatoxin-a hydrochloride (all >95% pure) and observed for 30 minutes following dosing (). LD_{50} values were determined to be 386 µg/kg (95% CI: 365-408) for (+)-anatoxin-a hydrochloride (0.32 mg anatoxin-a/kg) and 913 µg/kg (95% CI: 846-985) for racemic anatoxin-a hydrochloride (0.76 mg anatoxin-a/kg). No deaths or clinical signs occurred in mice treated with doses of (-)-anatoxin-a hydrochloride as high as 73 mg/kg (i.e., doses 189 times higher than (+)-anatoxin-a hydrochloride). The approximately 2-fold potency difference between (+)-anatoxin-a and the racemic mixture and the

lack of toxicity with (-)-anatoxin-a is consistent with mechanistic data indicating that (+)-anatoxin-a is the biologically active enantiomer.

An incompletely reported 2-day study in mice was performed to determine the doses for a neurodevelopmental study (Rogers et al., 2005). Doses of anatoxin-a (commercial product, >90% purity) in distilled water ranging from 10 to 400 µg/kg were administered by i.p. injection to female CD-1 mice for 2 consecutive days. Another study by the same laboratory identified the same commercial product and lot number as racemic (+/-)-anatoxin-a hydrochloride (MacPhail et al., 2005). Individual dose levels of anatoxin-a hydrochloride included 10, 100, 200, 250, 300 and 400 µg/kg (0.008, 0.08, 0.17, 0.21, 0.25 and 0.33 mg anatoxin-a/kg/day); however, it was not reported if these were the only levels tested. The authors noted that the study was conducted with 18 mice, but it is unclear if this refers to total number of animals or group size. Endpoints other than survival and clinical signs of toxicity were not evaluated. After one dose, mortality in the 0.08, 0.17, 0.21, 0.25 and 0.33 mg/kg groups was 0, 0, 50, 100 and 100%, respectively; all rats that lived to receive a second dose survived. Observations in mice administered lethal doses ($\geq$0.21 mg/kg-day) included decreased motor activity, altered gait, difficulty breathing and convulsions. The onset of clinical signs was noted after 5-6 minutes and death occurred within 10 minutes. Similar clinical signs (decreased motor activity level, altered gait and breathing difficulties) occurred at 0.17 mg/kg and in the 0.21 mg/kg mice that survived, but the convulsion stage was never reached and recovery occurred by 15-20 minutes. Additional information on this study, including results for doses lower than 0.08 mg/kg, was not reported.

7.2.1.2 Short-Term Studies

In the follow-up study to the 5-day study discussed in Section 7.2.2.1, groups of 10 male and 10 female Crl:CD-1(ICR)BR mice were administered aqueous (+)-anatoxin-a hydrochloride (commercial product, purity not reported) by gavage in daily doses of 0 (vehicle control), 0.12, 0.6 or 3 mg/kg (0, 0.1, 0.5 or 2.5 mg anatoxin-a/kg) for 28 days (Fawell and James, 1994; Fawell et al., 1999b). Endpoints that were examined included general condition and behavior (daily), body weight (weekly), food consumption (weekly), ophthalmic condition (final week), hematology (final week; erythrocyte count, packed and mean cell volumes, hemoglobin concentration, mean cell hemoglobin, mean cell hemoglobin concentration, total and differential leukocyte counts and platelet counts) and blood chemistry (final week; blood urea nitrogen, glucose, ALT, AST, total protein, albumin, albumin/globulin ratio, sodium, chloride, potassium, calcium, inorganic phosphorus, total bilirubin, creatinine and cholesterol). Gross pathology and organ weights (liver, kidneys, adrenals and testes) were evaluated in all animals at the end of the study. Comprehensive histological examinations were performed on the control and high dose groups, on animals that died or were sacrificed during the study and on gross lesions from all animals. Histology of the following tissues was evaluated: adrenals, aorta, brain, cecum, colon, duodenum, epididymis, eyes (including optic nerves), femur (including marrow), heart, jejunum, kidneys, liver (including gall bladder), lungs (including mainstem bronchi), mammary gland, mesenteric lymph node, esophagus, ovaries, pancreas, pituitary, prostate, rectum, salivary gland, sciatic nerve, seminal vesicles, skeletal muscle, skin, spinal cord, spleen, stomach, submandibular lymph node, testes, thymus, thyroid, parathyroid, trachea, urinary bladder and uterus.

There were three deaths during the course of the study. One death was not treatment-related: a male in the 0.1 mg/kg-day group was humanely sacrificed after being attacked by its cage mates. One 0.5 mg/kg-day male and one 2.5 mg/kg-day female died within 2.5 hours of dosing on days 10 and 14 of treatment, respectively. Both of these animals were clinically unremarkable prior to death, and the postmortem examinations were unable to establish the cause of death, leading the authors to conclude that a possible relationship to treatment could not be ruled out. The only other effects reported in treated animals were several minor hematology and blood chemistry changes that were not considered to be toxicologically significant. These alterations included statistically significant ($p<0.05$) increases in mean cell hemoglobin in males at >0.1 mg/kg-day, mean cell hemoglobin concentration in females at >0.5 mg/kg-day and serum sodium in females at ≥0.5 mg/kg-day. The alterations also included a non-significant increase in mean serum AST in males at ≥0.5 mg/kg-day and sporadic changes in a few other blood chemistry indices. The study authors concluded that the NOAEL was 0.1 mg/kg-day based on the two deaths that occurred at the higher dose levels. However, the authors state the NOAEL may actually be 2.5 mg/kg-day due to the low incidences of mortality that showed no dose-response or gender consistency (1/10 males at 0.5 mg/kg-day and 1/10 females at 2.5 mg/kg-day), the lack of characteristic clinical signs of acute neurotoxicity in the two animals that died, and the absence of toxicologically significant effects in the surviving mice, as well as the lack of effects at 2.5 mg/kg-day in mice as reported in the 5-day study discussed above and a developmental toxicity study (discussed below) (Fawell and James, 1994; Fawell et al., 1999b).

7.2.1.3 Subchronic Studies

Groups of 20 female Sprague-Dawley rats were administered anatoxin-a in the drinking water in concentrations of 0, 0.51, or 5.1 ppm for 7 weeks (Astrachan and Archer, 1981; Astrachan et al., 1980). The anatoxin-a used in this study was extracted from the culture media of *A. flos-aquae* (NRC-44-1) cells and partially purified; purity was not quantified, but the toxin had a UV absorbance spectrum that qualitatively indicated that anatoxin-a was the principal UV-absorbing component. The authors assumed that the test rats consumed 0.1 mL/g body weight-day (based on a preliminary water consumption study), indicating that the estimated daily intakes of anatoxin-a in the low and high dose rats were 0.05 and 0.5 mg/kg-day, respectively. Endpoints evaluated throughout the study included clinical signs, food consumption, body weight (weekly), red and total white blood cell counts (weekly) and serum enzyme activities (alkaline phosphatase, ALT, gamma glutamyl transpeptidase [GGT] and cholinesterase) (weekly). Endpoints assessed at the end of the exposure period included hepatic mixed function oxidase activity (aldrin epoxidation *in vitro*), measurement of post-sacrifice organ weights (liver, kidneys, spleen), gross pathology and histology (liver, kidneys, spleen, adrenals, heart, lungs and brain). No treatment-related effects were observed, suggesting an estimated NOAEL of 0.5 mg/kg-day (purity of extract tested not quantified).

7.2.1.4 Neurotoxicity

In a developmental toxicity study, groups of 8-11 time-pregnant CD-1 mice were administered (+/-)-anatoxin-a hydrochloride (commercial product, ≥90% purity) via i.p. injection in distilled water in doses of 0, 125, or 200 µg/kg-day (0, 0.10 or 0.17 mg anatoxin-a/kg-day) on GD 8-12 or 13-17 (Rogers et al., 2005). All mice were allowed to give birth and body weight and

viability of the pups were determined on postnatal days (PND) 1 and 6. The pups were subjected to a battery of neurotoxicity tests and the results discussed below.

Neurodevelopmental maturation was assessed by testing righting reflex, negative geotaxis and hanging grip time on PND 6, 12 and/or 20 in pups from dams exposed on GD 13-17. These behavioral tests were only conducted in the pups exposed on GD 13-17 because this gestational interval follows the onset of neurogenesis in the mouse brain (Rice and Barone, 2000). The litters from the dams exposed on GD 13-17 were normalized to eight pups (four males and four females) on PND 6, and a randomly selected male and female pup from each litter was evaluated on each test day. Righting reflex was tested on PND 6 and 12 and negative geotaxis was tested on PND 6, 12 and 20. Hanging grip time was tested on PND 12 and 20.

Almost all of the results of the righting reflex, negative geotaxis and hanging grip time tests showed no statistically significant differences between exposed and control groups or dose-related trends, indicating a lack of postnatal neurotoxicity. Findings in the righting reflex test included a non-significant ($p<0.086$) dose-related trend towards slower righting in males on PND 6 and a significantly (p value not reported) slower reflex in females than males in all treatment groups on PND 6 but no treatment or gender differences in righting reflex were observed on PND 12. The negative geotaxis test was complicated by control values (turning times) that did not decrease from PND 6 to 20 as expected and by many control and dose groups in which 1-4 pups fell off the screen before turning. Although the latter finding was a confounding factor because only data from mice that stayed on the inclined screen could be evaluated, there were no significant differences across treatment groups in either the number of fallen mice or the average turning times. There also were no treatment-related differences in hanging grip time on either test day. Hang time increased significantly from PND 12 to 20 in females, as expected although males did not show the expected increase in hang time. The investigators observed that gender differences are usually not evident at this age, indicating that random variability in the tested population may account for the finding in the male pups.

The mouse pups that were exposed to anatoxin-a on GD 13-17 in the Rogers et al. (2005) study were subsequently tested as adults to determine the effect of prenatal exposure to anatoxin-a on the motor activity of adult mice and their responses to nicotine challenge (MacPhail et al., 2005). Motor activity was measured in 30-minute sessions using a photocell device when the offspring were approximately 8 months old. Preliminary testing was performed in which groups of 12 male and 12 female mice were subcutaneously administered a single 0, 0.1, 0.3, 1.0 or 3.0 mg/kg dose of nicotine in saline approximately 5 minutes before testing motor activity. These mice were taken from litters that received saline vehicle on GD 8-12 or 13-17 (Rogers et al., 2005) and were assigned to the nicotine dose groups regardless of gestational period. Dose-related decreases in both horizontal and vertical activity were observed and 0.65 mg/kg was estimated to be the effective dose in 50% of subjects (ED_{50}) for nicotine in both sexes. Mice exposed to 0, 0.10, or 0.17 mg anatoxin-a/kg-day on GD 13-17 were then given the nicotine ED_{50} or saline vehicle approximately 5 minutes before testing motor activity. The nicotine ED_{50} and saline vehicle treatments were separated by 1 week. Group sizes were 10 per gender, except for the high-dose anatoxin-a female group, which contained nine mice. There were no differences in horizontal or vertical motor activity between the anatoxin-a-treated mice and the

controls. The report presents the results of the activity tests in bar graphs but provides no indication that the comparisons were based on a statistical evaluation of the data.

Additional information on neurobehavioral effects of anatoxin-a is available from intravenous and subcutaneous injection studies. Mice that were administered a single dose of (+)-anatoxin-a hydrochloride (commercial product, purity not reported) by intravenous injection were evaluated using the Irwin Screen and rota-rod tests at levels of 10-100 µg/kg (8-83 µg anatoxin-a/kg) and 30-60 µg/kg (25-50 g anatoxin-a/kg), respectively (Fawell and James, 1994; Fawell et al., 1999b). The Irwin Screen is a standard functional observational battery used to characterize CNS effects, including motor activity, behavioral changes, coordination and sensory/motor reflex responses. The rota-rod test assesses sensorimotor coordination by evaluating the animal's ability to remain on a rotating rod. There were no exposure-related effects in either of these tests although the highest doses caused clinical signs of neurotoxicity and death within 1 minute of exposure. The clinical signs of neurotoxicity included increased respiration, salivation, micturition, hyperactivity and Straub tail (contraction of the sacrococcygeus muscle, resulting in vertical erection of the tail).

Testing in rats that were administered aqueous (+)-anatoxin-a fumarate by subcutaneous injection showed that a single dose of 0.1 mg/kg (0.06 mg anatoxin-a/kg) caused decreased locomotor activity as well as a partial nicotine-like discriminative stimulus effect in animals trained to discriminate nicotine from saline in an operant conditioning procedure (Stolerman et al., 1992). As reported in an abstract, anatoxin-a also decreased response and reinforcement rates in multiple-schedule operant performance tests in rats treated by subcutaneous injection, although substantial tolerance developed upon repeated administration (Jarema and MacPhail, 2003).

7.2.1.5 Reproductive/Developmental Toxicity

In a developmental toxicity study, groups of 8-11 time-pregnant CD-1 mice were administered (+/-)-anatoxin-a hydrochloride (commercial product, ≥90% purity) via i.p. injection in distilled water in doses of 0, 125, or 200 µg/kg-day (0, 0.10 or 0.17 mg anatoxin-a/kg-day) on GD 8-12 or 13-17 (Rogers et al., 2005). All mice were allowed to give birth and body weight and viability of the pups were determined on postnatal days (PND) 1 and 6. Maternal toxicity was observed at 0.17 mg/kg-day, as shown by decreased motor activity immediately after treatment. The dams either died or completely recovered (Rogers et al., 2005). There were no effects on pup viability (number of live pups) on PND 1 or 6 in mice treated on GD 8-12 or 13-17 or on pup body weight on PND 1 or 6 in mice treated on GD 8-12. Pups treated on GD 13-17 showed a statistically significant dose-related trend for reduced body weight on PND 1 ($p<0.05$) but not on PND 6 ($p<0.07$). Body weight on PND 1 in the pups exposed on GD 13-17 was 7.1 and 8.7% less than controls at 0.10 and 0.17 mg/kg-day, respectively, and the differences between the treated and control groups were not significant. Although the trend data could have been interpreted as a treatment-related effect during the latter part of gestation, the investigators believed that the marginal effect on pup weight was due to random variability in litter size: the litter size of the GD 13-17 controls was noticeably smaller than the treated groups ($p=0.09$), and a difference in litter size would impact both birth weight and growth on PND 1-6 because pups

in smaller litters are larger at birth (McCarthy, 1967) and grow more rapidly postnatally (Rogers et al., 2003).

In a reproductive study, male mice were administered 50, 100, and 150 mg/kg/day anatoxin-a for seven consecutive days by i.p. (i.p.) route (Yavasoglu et al., 2008). Commercially available anatoxin-a fumarate was diluted in physiological saline (0.9%) and administered to 10 males in each treatment group. Although there were no significant changes in body weight gain and absolute and relative testes weights, a significant ($p < 0.01$) reduction in absolute and relative weights of cauda epididymis was observed in the 100 and 150 µg/kg treatment groups compared to control. Furthermore, a significant ($p < 0.01$), dose-dependent reduction in sperm count in the cauda epididymis was observed in all treatment groups compared to control. Histopathological examination of the testes revealed dose-dependent degeneration in seminiferous tubules, intercellular disassociation of spermatogenetic cell lines, sloughing of germ cells into the tubular lumen, vacuolization in Sertoli cells, and loss of germ cells. The epithelial thickness of seminiferous tubules decreased significantly in all treatment groups in a dose-dependent manner. The LOAEL was 50 µg/kg based on reduced sperm count in the cauda epididymis.

A developmental toxicity screening study was conducted in which groups of 10 and 12 pregnant Crl:CD-1(ICR)BR mice were administered aqueous (+)-anatoxin-a hydrochloride (commercial product, purity not reported) by gavage in doses of 0 (vehicle control) or 3 mg/kg-day (0 or 2.5 mg anatoxin-a/kg), respectively, on gestation days (GD) 6-15 (Fawell and James, 1994; Fawell et al., 1999b). Clinical signs and body weight were recorded until day 18 of gestation, at which time the maternal animals were sacrificed and necropsied. Developmental endpoints appear to have been limited to numbers of implantations (live and dead) and live fetuses, post implantation loss and fetal body weight, sex ratio and external abnormalities. No treatment-related maternal or fetal effects were observed although it was noted that mean fetal weight (male, female and total) in the treated group was marginally lower than in controls (data not reported). The lack of adverse effects in dams and fetuses identifies 2.5 mg/kg-day as a NOAEL for maternal and developmental toxicity.

A mammalian embryo toxicity test was conducted by Rodgers et al (2005) using CD-1 mouse embryos collected on GD 8. Cultured embryos (9-12) were exposed to 0.l, 1.0, 10, 25 µm anatoxin-a and evaluated for dysmorphogenesis. There was no significant dose-related increase in dysmorphogenesis produced by exposure to anatoxin-a at the concentrations tested. A perturbation in yolk sac vasculature (i.e. a decrease in large caliber vessels and a reduction in arborization) was observed in embryos exposed to 10 and 25 µm concentrations of anatoxin-a.

An embryo-larval toxicity test (AMPHITOX) was conducted by Rodgers et al (2005) using embryos beginning at Stage 18 (muscular response) or Stage 25 (complete operculum). Duplicate groups of ten embryos were placed in 5 cm glass petri dishes containing 10 mL of AS at 20°C. Stage 18 embryos were exposed to 0.03, 0.3, 3.0 or 30 mg L^{-1} anatoxin-a for 10 days. Stage 25 embryos were exposed to 30 mg L^{-1} anatoxin-a for 10 days. Embryos were monitored for viability and functional impairments over a period of 13 days. Results of anatoxin-a exposure in *Bufo arenarum* indicate the induction of a dose-dependent transient narcosis, with ≥ 70% of the embryos affected at the high dose in both exposure periods. Other adverse effects noted were edema and loss of equilibrium. Mortality occurred in both embryonic stages. In Stage 18, 20%

mortality was observed on day 8 in the highest dose group and reached 100% between days 10–13, 3 days after cessation of exposure. Mortality also occurred in the 0.3 and 3.0 ppm dose groups between days 10 and 13. Lethality in Stage 25 was initially observed at day 6 of exposure and had reached 100% by day 9.

In mammalian embryo culture studies, there was no evidence of adverse effects on postnatal growth or development following *in utero* exposure or embryotoxicity after *in vitro* exposure to anatoxin-a in the mouse. Preliminary data suggest that anatoxin-a exerts a narcotic effect on amphibian embryos and exposure results in delayed lethality.

7.2.1.6 Chronic Toxicity

No studies of the chronic effects of anatoxin-a in animals were identified as chronic studies are difficult to conduct due to the neurotoxic mode of action of anatoxin-a.

7.2.1.7 Carcinogenicity

There is no information on carcinogenicity in humans or animals or on possible carcinogenic processes and mode(s) of action for anatoxin-a.

7.2.2 Other Key Data

7.2.2.1 Mutagenicity and Genotoxicity

There is limited information regarding mutagenicity or genotoxicity of anatoxin-a. Preliminary findings by Sieroslawska and Rymuszka (2010) indicate anatoxin-a was genotoxic in an umuC assay with *Salmonella typhimurium* TA 1535/pSK1002. Anatoxin-a concentrations were 0.25, 0.5, 1 and 2µg/mL with a 2-hour exposure time to assess induction and expression of the umuC - *lacZ* reporter gene. The highest reported no-effect concentration of anatoxin-a without metabolic transformation was 0.25µg/mL. When S9 fraction was added to the samples, no effects were detected.

7.2.2.2 Immunotoxicity

No information regarding immunotoxicity of anatoxin-a was identified

7.2.2.3 Physiological or Mechanistic Studies

In vitro studies have demonstrated that (+)-anatoxin-a mimics the action of acetylcholine at neuromuscular nicotinic receptors (Aronstam and Witkop, 1981; Biggs and Dryden, 1977; Carmichael et al., 1975, 1979; Swanson et al., 1986) and is significantly more potent than acetylcholine and nicotine as an agonist. Anatoxin-a has become a very useful agent for investigating nicotinic acetylcholine receptors because it is resistant to enzymatic hydrolysis by acetylcholinesterase and because it is 100-fold more selective for nicotinic acetylcholine receptors than for muscarinic acetylcholine receptors (Aronstam and Witkop, 1981). Because

anatoxin-a is not degraded by cholinesterase or any other known cellular enzymes, muscle cells continue to be stimulated, causing muscular twitching, fatigue and paralysis. Severe overstimulation of respiratory muscles may result in respiratory arrest and rapid death, as observed in acute lethality studies in animals (Carmichael et al., 1975, 1977; Devlin et al., 1977; Stevens and Krieger, 1991b).

Anatoxin-a also acts as a nicotinic cholinergic agonist at receptors in the cardiovascular system of rats, resulting in increased blood pressure and heart rate (Adeyemo and Sirén, 1992; Dube et al., 1996; Sirén and Feuerstein, 1990), as well as in rat and human brain neurons (Durany et al., 1999; Thomas et al., 1993; Zhang et al., 1987). Anatoxin-a is a potent agonist of the secretory response of bovine adrenal chromaffin cells, presumably via neuronal-type nicotinic receptor activation (Molloy et al., 1995).

Anatoxin-a is capable of eliciting the release of neurotransmitters from presynaptic neuromuscular and brain cell terminals. Incubation of guinea pig ileum longitudinal muscle myenteric plexus preparations with anatoxin-a resulted in dose-dependent release of acetylcholine (Gordon et al., 1992). Anatoxin-a stimulated the release of dopamine from rat striatal synaptosomes in a dose-dependent manner (Clarke and Reuben, 1996; Rowell and Wonnacott, 1990; Soliakov et al., 1995; Wonnacott et al., 2000). These findings indicate that anatoxin-a can bind to presynaptic nicotinic receptors to trigger neurotransmitter release and contribute to increased stimulation of postsynaptic receptors.

7.2.2.4 Structure-Activity Relationship

Anatoxin-a is produced as the natural stereoisomer, (+)-anatoxin-a, by some strains of Anabaena (particularly *A. flos-aquae*) and at least four other genera of freshwater cyanobacteria, including Aphanizomenon, Microcystis, Planktothrix and Oscillatoria (Devlin et al., 1977; Fawell et al., 1999b; Huber, 1972; Viaggiu et al., 2004). As discussed above, (+)-anatoxin-a is a nicotinic acetylcholine receptor agonist that exerts its effects at both peripheral and central sites in the nervous system. It is generally believed that nicotinic agonists form hydrogen bonds in the planar region and contain a bulky cationic group approximately 5.9 Å from the hydrogen bond (Beers and Reich, 1970; Chothia and Pauling, 1970; Spivak and Albuquerque, 1982); anatoxin-a exhibits these characteristics. The importance of stereospecificity was demonstrated in assays of contracture potency in frog rectus abdominis muscle preparations; natural (+)-anatoxin-a exhibited at least a 2.5- and 150-fold greater potency than racemic and (-)-anatoxin-a, respectively (Spivak et al., 1983; Swanson et al., 1986). Similar potency differences were demonstrated in *in vivo* lethality assays in mice (Valentine et al., 1991). As discussed in Section 7.2.2.4, acute i.p. LD_{50} values of 386 and 913 µg/kg were determined for (+)-anatoxin-a hydrochloride and racemic anatoxin-a hydrochloride, respectively. No clinical signs or deaths occurred in mice that were similarly treated with doses of (-)-anatoxin-a hydrochloride as high as 73 mg/kg. These findings indicate that (+)-anatoxin-a was 2.4 and at least 189 times as potent as racemic and (-)-anatoxin-a, respectively.

Anatoxin-a is significantly more potent than acetylcholine and nicotine as an agonist at neuromuscular nicotinic acetylcholine receptors. Anatoxin-a has been shown to bind to the nicotinic acetylcholine receptor with an affinity approximately 3.6 times greater than

acetylcholine (Swanson et al., 1986). Following complete inhibition of acetylcholinesterase activity in frog rectus abdominis muscle preparations, anatoxin-a exhibited an 8-fold greater potency by contracture than acetylcholine (Swanson et al., 1986). Anatoxin-a was 7-136 times more potent than nicotine in a series of *in vitro* acetylcholine receptor (guinea pig ileum, rat phrenic nerve, chick biventer cervicis muscle) and mouse intravenous (neuropharmacological signs) screening studies (Fawell and James, 1994; Fawell et al., 1999b). Additionally, the agonist potency of (+)-anatoxin-a was 3-50 times more than nicotine and approximately 20 times more than acetylcholine at neuronal nicotinic acetylcholine receptors from rat hippocampal synaptosomes, fetal rat hippocampal neurons, mouse M10 cells and frog (*Xenopus*) oocytes (Thomas et al., 1993).

Commented [IS58]: Are these models of isolated cells, i.e. *in vitro* as described, or are they *ex vivo* tests?

Neuromuscular and neuronal assays of structure activity relationships indicate that N-methylation of anatoxin-a greatly reduces the acetylcholine-mimicking effect at nicotinic cholinergic receptors (Aracava et al., 1987; Costa et al., 1990; Kofuji et al., 1990; Stevens and Krieger, 1990; Swanson et al., 1989, 1991; Wonnacott et al., 1991).

7.2.3 Hazard Characterization

7.2.3.1 Synthesis and Evaluation of Major Noncancer Effects

The main known toxic effect of anatoxin-a is acute neurotoxicity that is manifested as progressive clinical signs that include loss of coordination, muscular fasciculations, convulsions and death by respiratory paralysis. It is well documented that anatoxin-a acts by mimicking the action of acetylcholine at neuromuscular nicotinic receptors (Aronstam and Witkop, 1981; Biggs and Dryden, 1977; Carmichael et al., 1975, 1979; Swanson et al., 1986). As an agonist that is significantly more potent than acetylcholine and is not degraded by cholinesterase, (+)-anatoxin-a interacts with the nicotinic acetylcholine receptors to cause persistent stimulation of muscle cells (Swanson et al., 1986; Thomas et al., 1993).

A limited amount of information is available on the health effects of anatoxin-a in humans. Several cases of nonlethal human poisonings caused by ingestion of lake water containing *Anabaena sp*. have been reported and the most prominent and best documented effects were acute gastrointestinal disorders. In a more recent report, anatoxin-a was implicated in the poisonings of two teenage boys who swallowed water from a pond containing an algal bloom (Behm, 2003). One of the boys suffered a seizure and died from heart failure 2 days after swallowing the water, and the other boy became sick with severe diarrhea and abdominal pain but survived. Testing of stool samples from both boys revealed the presence of *A. flos-aquae* cells, and analyses of blood, liver tissue and ocular fluid from the boy who died found a compound initially identified as anatoxin-a. A definitive diagnosis of anatoxin-a as the cause of death was confounded by the apparent delay between exposure and overt toxicity (Behm, 2003), and subsequent analysis determined that the detected compound was not anatoxin-a (Carmichael et al., 2004). Relevant dose-response information, including estimated amounts of water or toxin ingested, was not provided in either of the above reports. Anatoxin-a has also been implicated in cases of animal poisonings following consumption of water containing blooms of *A. flos-*

aquae (Carmichael and Gorham, 1978; Edwards et al., 1992; Gunn et al., 1992; Pybus et al., 1986) although no quantitative exposure data are available.

Information on the *in vivo* effects of anatoxin-a in orally-exposed laboratory animals is available from several acute and short-term studies and one subchronic study that provide a limited amount of dose-response data on systemic and developmental toxicity. These studies are summarized in table 7-15. Acute toxicity data for anatoxin-a are limited to the results of lethality assays in mice that determined a single-dose LD_{50} value of 13.3 mg anatoxin-a/kg and identified neurotoxicity as the cause of death (Fitzgeorge et al., 1994; Stevens and Krieger, 1991b).

Information on the short-term oral toxicity of anatoxin-a is available from 5- and 28-day systemic toxicity studies in mice and a developmental toxicity study in mice (Fawell and James, 1994; Fawell et al., 1999b). The 5-day mouse study used four dose levels (1.2, 2.5, 6.2 and 12.3 mg/kg-day by gavage) but is limited by small numbers of animals (2/sex/dose), lack of controls and few endpoints (clinical signs, body weight, food consumption, and necropsy). Based on dose-related mortality at 6.2 mg/kg-day (1/4 mice) and 12.3 mg/kg-day (4/4 mice), the NOAEL is 2.5 mg/kg-day.

The 28-day toxicity study in mice (Fawell and James, 1994; Fawell et al., 1999b) is the best-designed short-term oral study of anatoxin-a available in the literature. This study identified 2.5 mg/kg-day as the NOAEL for systemic toxicity and is supported by the 2.5 mg/kg-day NOAEL in the 5-day mouse study based on maternal effects and developmental toxicity in mice (Fawell and James, 1994; Fawell et al., 1999b).

Information on the subchronic oral toxicity of anatoxin-a is available from a 7-week drinking water study in rats (Astrachan and Archer, 1981; Astrachan et al., 1980). This study is limited by the use of only two dose levels (0.05 and 0.5 mg/kg-day) and non-comprehensive examinations, particularly for hematology (two indices), blood chemistry (four serum enzymes) and histology (seven tissues). No treatment-related effects were found at 0.5 mg/kg-day however the small number of endpoints and lack of an adverse effect level do not provide adequate information to support development of a subchronic toxicity threshold.

7.2.3.2 Synthesis and Evaluation of Carcinogenic Effects

No information regarding carcinogenicity in humans or animals or on possible carcinogenic precursor effects were identified.

7.2.3.3 Weight of Evidence Evaluation for Carcinogenicity

In accordance with the Guidelines for Carcinogen Risk Assessment (U.S. EPA, 2005a), the weight of evidence descriptor for the carcinogenic hazard potential of anatoxin-a is "Inadequate Information to Assess Carcinogenic Potential."

Table 7-15. Summary of Noncancer Results in Animal Studies of Oral Exposure to Anatoxin-a

Species	Sex	Average Daily Dose (mg/kg-day)	Exposure	NOAEL (mg/kg-day)	LOAEL (mg/kg-day)	Responses	Comments	Reference
Acute Exposure								
No suitable studies available								
Short-Term Exposure								
Mouse	M,F	1.2, 2.5, 6.2,12.3 (gavage with commercial anatoxin-a)	5 days	2.5	6.2	Effect level of 6.2 mg/kg-day due to mortality in the two highest dose groups.	No clinical signs or effects on body weight, food consumption, survival or necropsy at <2.5 mg/kg-day. Other endpoints not examined. No control group and small group sizes (2/sex/dose).	Fawell and James, 1994; Fawell et al., 1999b
Mouse	M,F	0, 0.1, 0.5,2.5 (gavage with commercial anatoxin-a)	28 days	2.5	ND	Two deaths at >0.5 mg/kg-day; it was unclear whether these deaths could be attributed to compound administration. Minor hematology and blood chemistry changes at >0.1 mg/kg-day were not clearly exposure-related and/or toxicologically significant.	Well-designed study that investigated clinical signs, body weight, food consumption, ophthalmic condition, hematology, blood chemistry, gross pathology, organ weights and histology 10 mice/sex/dose.	Fawell and James, 1994; Fawell et al., 1999b
Mouse	F	0, 2.5 (gavage of commercial anatoxin-a)	9 days; gestation days 6-15	2.5	ND	No exposure-related adverse maternal or fetal effects. Mean fetal weight was marginally reduced at 2.5 mg/kg-day (data not reported)	Maternal endpoints included clinical signs, body weight and necropsy. Developmental endpoints included numbers of implantations and live	Fawell and James, 1994; Fawell et al., 1999b

Species	Sex	Average Daily Dose (mg/kg-day)	Exposure	NOAEL (mg/kg-day)	LOAEL (mg/kg-day)	Responses	Comments	Reference
						but not considered to be toxicologically significant.	fetuses, post implantation loss, body weight, sex ratio and external abnormalities. No fetal internal examinations. 10-12 mice/dose.	
Sub-chronic Exposure								
Rat	F	0, 0.05, 0.5 (extract from *A. flos-aquae* provided in drinking water)	7 weeks	0.5	ND	None	Limited number of endpoints: clinical signs, food consumption, body weight, red and total white blood cell counts, serum enzyme activities (alkaline phosphatase, ALT, GGT, cholinesterase), hepatic MFO activity (aldrin epoxidation *in vitro*), organ weights (liver, kidneys, spleen), gross pathology and histology (liver, kidneys, spleen, adrenals, heart, lungs and brain). 20 rats/dose.	Astrachan and Archer, 1981; Astrachan et al., 1980

7.2.3.4 Potentially Sensitive Populations

There is no information on the degree to which children might differ from adults in the disposition of, or response to, anatoxin-a. Likewise, there is no information on possible gender differences in the disposition of, or response to, anatoxin-a.

It is noted that anatoxin-a may interact with anticholinergic agents. Anticholinergic agents have been recommended for the treatment of various medical conditions, but therapeutic uses are mainly in four areas: atony of the smooth muscle of the intestinal tract and urinary bladder, glaucoma, myasthenia gravis and termination of the effects of competitive neuromuscular blocking agents (Taylor, 1996). It is conceivable that people using anticholinergic agents for therapeutic purposes could be at risk of experiencing an increase in unwanted side effects if exposed to anatoxin-a due to the potential for additivity of adverse effects.

7.3 Cylindrospermopsin

7.3.1 Animal Studies

7.3.1.1 Acute Toxicity

Oral Exposure

Twelve male MF1 mice were administered a saline suspension of freeze-dried *C. raciborskii* cells (strains PHAWT/M or PHAWT/1) by gavage in single reported doses ranging from 4.4 to 8.3 mg/kg (cylindrospermopsin-equivalent), and observed for the following 8 days (Seawright et al., 1999). The following dose levels were tested (one mouse per level except as noted): 4.4, 5.3, 5.7 (two mice), 5.8, 6.2, 6.5, 6.7, 6.8, 6.9, 8.0 and 8.3 mg/kg; there was no control group. Eight of the 12 mice died. The lowest lethal dose was 4.4 mg/kg, the highest nonlethal dose was 6.9 mg/kg and the average lethal dose was approximately 6 mg/kg. Deaths occurred 2-6 days after treatment, and histological examinations showed effects that included fatty liver with periacinar coagulative necrosis, acute renal tubular necrosis, atrophy of the lymphoid tissue of the spleen and thymus, subepicardial and myocardial hemorrhages in the heart and ulceration of the esophageal section of the gastric mucosa. Some of the animals also developed thrombohemorrhagic lesions in one or both eye orbits.

An aqueous suspension of a cell-free extract of freeze-dried and sonicated *C. raciborskii* cells (strain AWT 205) was administered to an unspecified number of male Swiss mice in a single gavage dose of 1400 mg extract/kg (Falconer et al., 1999). The cylindrospermopsin content of the extract was not specified, but ranged from 1.3 to 5.4 mg/g extract in concurrent i.p. experiments, indicating that the cylindrospermopsin-equivalent gavage dose was likely in the range of 1.8-7.6 mg/kg. This dose level was not fatal, but the authors observed severe liver and kidney pathology. Additional information on the design and results of the oral study were not provided.

Another gavage study reported that the minimum oral lethal dose of a saline extract of freeze-dried *C. raciborskii* cells (strain AWT 205) in male Swiss albino mice was 2500 mg extract/kg (Falconer and Humpage, 2001). Fifty three (53) mice were treated up to three times orally with the *C. raciborskii* extract and monitored for 30 weeks. Following euthanization, five tumors were found in the 53 treated mice when major organs were examined histologically. Based on a reported cylindrospermopsin content of 5.5 mg/g extract determined by HPLC, the equivalent lethal dose of cylindrospermopsin was 13.8 mg/kg.

Groups of four Quackenbush mice were administered a cell-free extract of freeze-dried and sonicated *C. raciborskii* cells (strain AWT 205) in water in a single gavage dose of 0, 1, 2, 4, 6 or 8 mg cylindrospermopsin/kg and observed for the following 7 days (Shaw et al., 2000, 2001). All animals were evaluated for gross pathological and histological (liver, kidney, spleen, heart, lungs and thymus) changes. Hepatic effects were observed at all dose levels, as shown by foamy hepatocellular cytoplasmic changes at 1 and 2 mg/kg, lipid infiltration with some hepatocyte necrosis in the periacinar region at 4 mg/kg, and uniformly pale and mottled livers with lipid infiltration throughout and cell necrosis mainly in the periacinar region at 6 mg/kg. Mortality occurred in 2/4 mice at 6 mg/kg (in 5 days) and 4/4 mice at 8 mg/kg (in 24-48 hours). Additional information on the experimental design and results was not reported.

I.p. Exposure

Acute i.p. lethality values have been determined for cylindrospermopsin purified from extracts of cultured *C. raciborskii* or *U. natans* cells (Ohtani et al., 1992; Shaw et al., 2000, 2001; Terao et al., 1994). In male CH3 mice, 24-hour and 5- to 6-day LD_{50} values of 2.1 and 0.2 mg/kg, respectively, have been reported for a single i.p. dose of purified cylindrospermopsin (purity not reported) (Ohtani et al., 1992). Another study found that a single 0.2 mg/kg i.p. dose of purified cylindrospermopsin (purity not reported) caused 50% moribundity after 31 hours in Quackenbush mice (Shaw et al., 2000, 2001). The main pathological findings in the moribund animals were lipid infiltration and cell necrosis in the liver. Terao et al. (1994) found that the liver was the main target of toxicity in male ICR mice administered a single 0.2 mg/kg i.p. dose of purified cylindrospermopsin (purity not reported); treatment-related lesions were also noted in the thymus, kidney and heart. Ultrastructural tissue examinations identified four sequential phases of liver damage: inhibition of protein synthesis, membrane proliferation, fat droplet accumulation and cell death.

The results of acute i.p. studies of extracts of freeze-dried and sonicated *C. raciborskii* cells are generally similar to those of the i.p. studies of purified cylindrospermopsin. A single 0.2 mg/kg cylindrospermopsin-equivalent dose caused 50% moribundity in Quackenbush mice after 98 hours (Shaw et al., 2000, 2001). Other single-dose LD_{50} values, expressed as cylindrospermopsin-equivalent doses included 24-hour and 7-day values of 0.29 and 0.18 mg/kg, respectively, in male Swiss mice (Hawkins et al., 1997). The 24-hour LD_{50} was lower than the 24-hour i.p. LD_{50} of 2.1 mg/kg for purified cylindrospermopsin in mice (Ohtani et al., 1992), leading the authors to suggest that the extract contained more than one toxin. Although the liver was the main target organ in the extract studies, lesions also occurred in the kidney, adrenal gland, lung, and intestine (Hawkins et al., 1985, 1997; Shaw et al., 2000, 2001).

A single dose i.p. LD_{50} value of 64 mg freeze-dried culture/kg was determined in mice observed for 24 hours (Hawkins et al., 1985). Falconer et al. (1999) assessed the acute lethality and liver and kidney effects of four different batches of cell-free extracts of sonicated freeze-dried *C. raciborskii* cells in male Swiss albino mice treated by single i.p. injection. Reported 24-hour and 7-day LD_{50} values for the four batches were 50-110 and 20-65 mg extract/kg, respectively. The cylindrospermopsin content in the four batches varied from 1.3 to 5.4 mg/g extract, indicating that the cylindrospermopsin-equivalent LD_{50} values were 0.07-0.6 mg/kg (24-hour) and 0.03-0.4 mg/kg (7-day). Liver damage was characterized by cellular vacuolation, intercellular spaces and dark nuclear and cytoplasmic staining. Kidney damage included proximal tubule epithelial necrosis and presence of proteinaceous material in the distal tubules. There was no clear correlation between cylindrospermopsin batch concentration and the LD_{50} values or severity of liver or kidney lesions, leading the study authors to suggest that more than one toxin was present in the extract.

7.3.1.2 Short Term Studies

Oral Exposure

Groups of four Quackenbush mice were administered purified cylindrospermopsin by daily gavage for 14 days (Shaw et al., 2000, 2001). The cylindrospermopsin was purified (purity not reported) from an extract of freeze-dried *C. raciborskii* cells (strain AWT 205). All animals were evaluated for gross pathological and histological (liver, kidney, spleen, heart, lungs and thymus) changes. The authors identified the following effect levels: a NOAEL of 0.05 mg cylindrospermopsin/kg-day and a LOAEL of 0.15 mg cylindrospermopsin/kg-day for lipid infiltration in the liver, and a NOAEL of 0.3 mg cylindrospermopsin/kg-day (highest tested dose) for lymphophagocytosis in the spleen. Additional data on the experimental design and results was not reported by the authors.

Six Quackenbush mice and two Wistar rats were exposed for 21 days to drinking water containing 800 µg/L cylindrospermopsin (Shaw et al., 2000, 2001). The water was "sourced" from a dammed impoundment. The reported approximate daily dose based on water consumption was 0.2 mg cylindrospermopsin/kg-day in both species. Gross pathological and histological (liver, kidney, spleen, heart, lungs and thymus) examinations showed no effects, indicating that 0.2 mg/kg-day was a NOAEL in the rats and mice. Additional information on the experimental design and results was not reported by the authors.

Other Routes of Exposure

Chernoff et al. (2011) conducted a series of studies to evaluate the effects of cylindrospermopsin treatment during different gestational periods primarily focusing on effects in the dams and not the pups. In the first study, pregnant CD-1 mice were administered daily i.p. injections of 0.05 mg/kg cylindrospermopsin (purified from bulk cultures of *C. raciborskii* and analyzed to be >98% pure) for 5 days on gestational day (GD) 8-12 (42 animals) or GD 13-17 (42 animals). Controls received distilled water (i.p.) on GD 8-12 (26 animals) or GD 13-17 (26 animals). Two to three control dams and 3-5 cylindrospermopsin exposed dams were sacrificed the day following the last dose and on post-treatment days 7 and 14 for both exposures; days 28

and 42 for the GD 8-12 exposure and on days 35 and 49 for the GD 13-17 exposures. Blood, liver and kidney samples were obtained at each time point for further analyses. Endpoints measured included maternal weight, clinical signs of toxicity, during and after the dosing period; serum chemistries indicative of hepatic and/or renal function and general homeostasis, histopathology of liver and kidney tissues, and hepatic gene expression after the dosing period.

Fifty-five percent of treated dams had died by GD13 compared to none in the controls. Vaginal bleeding occurred in 13 of the pregnant mice. Other clinical signs observed occasionally in cylindrospermopsin treated dams included orbital and tail bleeding. Maternal body weight gain was reduced throughout treatment. Mice sacrificed the day after dosing ended had elevations in numerous serum enzymes (ALT, AST, alpha-1-antitrypsin, sorbitol dehydrogenase (SDH), and LDH only 2-5 mice per assay). However, levels returned to control levels by day 7 after exposure. Blood urea nitrogen (BUN) and creatinine (indicators of kidney damage) were significantly increased the day after exposure ended, but had returned to control levels 7 days after exposure. There were no significant differences in relative kidney or liver weights at any time. Histopathology revealed an increase in the incidence of liver necrosis (1/9 in controls and 7/19 treated animals) and an increase in the incidence of nephrosis and/or renal inflammation 0/9 in controls and 5/19 treated animals) in mice treated on GD 8-12, normal within 7 days of the last exposure.

A tissue sample adjacent to that collected for histopathology was obtained from the liver for gene array analysis. RNA was purified, labeled and analyzed by a two-channel custom-spotted microarray developed for evaluation of liver toxicity by the EDGE Program, University of Wisconsin. Real time RT-PCR analysis was performed using genes selected to verify the effects noted by the microarray analysis. These analyses were conducted using the same RNA samples utilized for microarray analysis with the addition of either 2 or 3 samples per group from the GD13-17 dose group that were included to evaluate treatment recovery at 7, 16, 28 days post dosing. Results indicate that expression of genes involved in ribosomal biogenesis, xenobiotic and lipid metabolism, inflammatory response, and oxidative stress were altered over 2 weeks after treatment ended and returned to normal by 4 weeks.

In the second study, 42 pregnant CD-1 mice were administered daily i.p. injections of 0.05 mg/kg/ day cylindrospermopsin (purified from bulk cultures of *C. raciborskii* and analyzed to be >98% pure) on GD 13-17 (Chernoff et al., 2011). Controls (26) received distilled water (i.p.) on GD 13-17. Some animals (2-3 controls and 3-5 cylindrospermopsin exposed) were sacrificed the day after the last dose and on day 7, 14, 35 or 49 after exposure. None of the pregnant dams treated on GD 13-17 died. Unlike the dams treated during GD 8-12, there was no vaginal bleeding. However, gastrointestinal bleeding was noted in 3 of the dams either on Day 1 or 7 after exposure.

Maternal body weight gain was not significantly affected. Mice sacrificed the day after dosing ended had elevations in ALT, AST, alpha-1-antitrypsin, SDH, and LDH (2-5 mice per assay). Levels returned almost to control levels within 7 days. BUN was slightly, but significantly, increased the day after exposure, but returned to control levels 7 days after exposure. Relative liver weight was only significantly increased 7 days after dosing. Histopathology revealed an increase in the incidence of liver necrosis. Tissue histopathology

features were normal at the time of the 7 days post treatment sacrifice. Expression of genes involved in ribosomal biogenesis, xenobiotic and lipid metabolism, inflammatory response, and oxidative stress were altered for 2 weeks after treatment ended and returned to normal by 4 weeks.

7.3.1.3 Subchronic Studies

Oral Exposure

Groups of male Swiss albino mice (10 per dose, 6 in the highest dose group) were administered purified cylindrospermopsin in water by gavage in doses of 0, 30, 60, 120 or 240 µg/kg-day for 11 weeks (Humpage and Falconer, 2003). The cylindrospermopsin was purified (purity not reported) from an extract of freeze-dried *C. raciborskii* cells (strain AWT 205). Endpoints monitored throughout the study included food and water consumption and body weight. A clinical examination focused on physiological and behavioral signs of toxicity was conducted after 9 weeks of exposure and other endpoints monitored were:

- hematology (all animals; red cell counts, hemoglobin, packed cell volume, and white cell total and differential counts),
- serum chemistry (five mice/group except all six mice at the high dose; total protein, albumin, globulin, glucose, creatinine, urea, total bilirubin, total bile acids, cholesterol, triglycerides, sodium, potassium, calcium, bicarbonate, creatinine kinase, alanine and aspartate aminotransferases [ALT and AST, respectively] and alkaline phosphatase (ALP) and
- urine (five mice/group excluding high dose; specific gravity, protein, glucose, ketones, creatinine, sodium, potassium, chloride, calcium, bicarbonate, phosphate, pH, volume and presence of blood).

All evaluations were conducted near or at the end of the treatment period.

Postmortem examinations included organ weights (liver, spleen, kidneys, adrenal glands, heart, testis, epididymis and brain) and comprehensive histological evaluations. The histological examinations were conducted in accordance with the Organization for Economic Cooperation and Development (OECD) recommendations and performed on the following tissues: liver, kidney, heart, lungs, thymus, thyroid, trachea, salivary glands, adrenal glands, epididymis, testis, prostate, gall bladder, esophagus, stomach, duodenum/small intestine, large intestine, pancreas, spleen, urinary bladder, eyes, lymph nodes, aorta, cerebrum, cerebellum, spinal cord (cervical, thoracic and lumbar) and peripheral nerve.

No deaths were reported in mice exposed to purified cylindrospermopsin under the study conditions. The mean final body weight was 7-15% higher than controls in all dose groups, but the increases were not dose-related and were statistically significant only at 30 and 60 µg/kg-day (Humpage and Falconer, 2003). There were no significant changes in food consumption; however, water intake was significantly reduced in all dose groups (data not reported). Relative kidney weight was increased in a significant, dose-related manner beginning at 60 µg/kg-day (12-23% greater than controls) and relative liver weight was significantly increased only at the high dose of 240 µg/kg-day (13% greater than controls). Information on absolute kidney and liver weights was not reported. Absolute testis weights were significantly increased at >60

µg/kg-day (data not reported). The organ weight changes were not significant when normalized to body weight.

The hematology, serum chemistry and urine evaluations showed no clear exposure-related changes in any endpoint (including serum indicators of liver injury), except for significant decreases in urine protein concentrations (g/mmol creatinine) at >120 µg/kg-day and urine specific gravity at 240 µg/kg-day (data presented graphically). The postmortem examinations showed "minor increases in histopathological damage to the liver" at >120 µg/kg-day and proximal renal tubular damage at 240 µg/kg-day, but additional information regarding the type, severity and incidences of the liver and kidney lesions was not reported.

Although cylindrospermopsin has been shown to inhibit protein synthesis in the liver, serum albumin, a major product of liver protein synthesis, was not decreased in this study (Humpage and Falconer, 2003). The most sensitive effects observed were dose related decreased urinary protein at >120 µg/kg-day and increased relative kidney weight at >60 µg/kg-day. The noted decrease in urinary protein excretion reflects a specific effect of cylindrospermopsin on protein synthesis, as well as the possibility that the cyanotoxin impacts a functional change in the nephron. Effects on the kidney and urine protein levels observed in male mice in both drinking water and gavage studies were observed; however, the biological relevance needs further investigation. Because the renal effects observed by Humpage and Falconer (2003) are consistent with a known mode of action of cylindrospermopsin, and plausibly represent part of the progression of effects leading to toxicity, they are considered to be adverse. This study identifies a NOAEL and LOAEL of 30 and 60 µg/kg-day, respectively.

The potential effects of cylindrospermopsin were investigated in a 42-week mouse oral dose step-up study by Sukenik et al. (2006). Four-week weaned male and female ICR mice (initial body weight 24–28 g) were supplied with food and water *ad libitum* throughout the study. Animals were divided into two groups consisting of 20 males and 20 females in each group. The mice of the control group received freshly prepared cyanobacterial growth medium in their drinking water, whereas mice of the experimental group received spent medium that contained several different concentrations of cylindrospermopsin obtained from actively grown cultures of *Aphanizomenon ovalisporum* (isolated from Lake Kinneret during a 1994 bloom). Cylindrospermopsin concentrations in the spent medium and in drinking water provided to experimental animals were quantified by HPLC. The concentration of cylindrospermopsin in drinking water was increased gradually from 100 to 550 µg/L (100 µg/L for weeks 0-8, 200 µg/L for weeks 8-16, 350 µg/L for weeks 16-24, and 550 µg/L for weeks 24-42). The daily intake of the toxicant by animals in the experimental group was 10 µg/kg for weeks 0-8 for males and females; for weeks 8-16, 15 µg/kg for males and 17 µg/kg for females; for weeks 16-24, 30 µg/kg for males and 34 µg/kg for females; and weeks 24-42, 48 µg/kg for males and 55 µg/kg for females. Results showed no effect on any organ weight/body weight ratios but did indicate hematocrit levels were significantly ($p < 0.05$) elevated in both male and female mice after 16 weeks of exposure to cylindrospermopsin in drinking water. The observed changes in the hematocrit level were accompanied by deformation of RBCs. After 20 weeks' exposure to low concentrations (daily dose between 10 and 20 µg/L), many RBCs changed into acanthocyte-like cells, a form of red-blod cell that has a spiked thorn-like cell membrane. Longer exposure for an additional 22 weeks to higher daily doses (up to 50 µg/L) left only a few normal RBCs as almost

all RBCs turned into acanthocytes. Reisner et al (2004) observed acanthocyte-like cells in in mice exposed to 0.6 mg/L (66 µg/kg) cylindrospermopsin in drinking water for three weeks. The cylindrospermopsin source was the same as in the Sukenik 2006 study but purified on a HPLC column (purity not provided). A significant ($p < 0.05$) increase in relative liver weight (after 42 weeks) and kidney weight (after 20 and 42 weeks) was observed in male and female mice in the cylindrospermopsin treatment groups. Based on the structural changes in mice RBC that likely lead to increased hematocrit, Sukenik et al proposed a LOAELof 20 µg/kg/day for cylindrospermopsin in drinking water.

In another study, groups of male Swiss albino mice (10 per dose except 12 controls and 5 at high-dose) were exposed to a cell-free extract of sonicated and frozen *C. raciborskii* cells (strain AWT 205) in the drinking water at cylindrospermopsin doses of 0, 216, 432 or 657 µg/kg-day for 10 weeks (doses based on actual water consumption) (Humpage and Falconer, 2003). Food and water consumption and body weight were measured throughout the study. Urinalysis (12 unspecified parameters) was performed after 5 and 10 weeks. Serum chemistry (15 unspecified parameters) evaluations and examinations of unspecified major organs (organ weight, gross pathology and histopathology) were performed at the end of the exposure period. Hematology was not evaluated.

Final body weights were significantly reduced at 432 and 657 µg/kg-day (9 and 7% less than controls, respectively), and relative liver and kidney weights were significantly increased in a dose-related manner at 216-657 µg/kg-day (27-47 and 30-43% greater than controls, respectively). Other statistically significant effects included increased serum total bilirubin at >216 µg/kg-day, decreased serum total bile acids at >216 µg/kg-day and decreased urine protein concentration (g/mmol creatinine) at >432 µg/kg-day. There were no clear exposure-related changes in any other serum or urine endpoints and no additional indicators of liver or kidney injury. Results of the postmortem pathology examinations were not reported. The lowest dose of 216 µg/kg-day represents a LOAEL for this study, based on increased relative liver and kidney weights, increased serum bilirubin, and decreased serum bile acids. An increase in serum bilirubin is indicative of liver dysfunction or bile duct blockage. Serum bile acids can be decreased due to an inhibition of bile acid synthesis or an interference with bile acid resorption from the gastrointestinal tract; bile acids are synthesized from cholesterol in the liver, conjugated, excreted in the bile and resorbed from the ileum.

In a third study, Quackenbush mice were administered drinking water containing a cell-free extract of freeze-dried and sonicated *C. raciborskii* cells (strain AWT 205) for 90 days (Shaw et al., 2000, 2001). Gross pathological and histological (liver, kidney, spleen, heart, lungs and thymus) examinations showed no effects at dose levels as high as 0.15 mg/kg-day (the highest tested dose). Additional information on the experimental design and results were not reported in the study.

7.3.1.4 Neurotoxicity

The published literature indicates exposure to cylindrospermopsin does not result in a neurotoxic response.

7.3.1.5 Developmental/Reproductive Toxicity

A teratology study with purified cylindrospermopsin was conducted with the CD-1 mouse to investigate developmental toxicity in mammals (Rogers et al, 2007). The study was comprised of three experiments. The first was a standard teratology study that utilized a series of dose levels and exposed pregnant females to cylindrospermopsin on GD 8-12 by the i.p. (i.p.) route. Purified cylindrospermopsin (> 98%) was supplied by the Australian Water Quality Centre and administered in distilled water by i.p. injection during the study. In experiment 1 dosing to groups of 20-25 pregnant females on GD 8 to 12 at treatment levels of 0, 8, 16, 32, 64, 96 and 128 µg/kg. For experiments 2 and 3, there were 2 groups per exposure period evaluated with 23-51 mice per group. Results 1 indicate increased mortality in mice exposed to 32 µg/kg or higher. A significant ($p < 0.01$ to 0.05) dose-related increase in liver-to-body-weight ratio was observed in the dams from the 8, 16 and 32 µg/kg dose groups compared with control. Fetal weight and mortality were not significantly affected in the 32 µg/kg treatment group or in litters from the small number of pregnant animals surviving in the 64 and 128 µg/kg dose groups. No adverse effects on average litter size, fetal weight or incidence of anomalies were observed.

In experiments 2 and 3, 2 groups per exposure period were evaluated for postnatal effects on their pups. The dams (23-51 mice per group) were dosed i.p. (50 µg/kg daily) on GD 8 to 12 (experiment 2) and GD 13 to 17 (experiment 3) and litters were monitored for postnatal growth. Significant ($p < 0.01$ to 0.05) reductions in litter size were observed in both the GD 8 to 12 and GD 13 to 17 treated groups. Average pup weight was significantly ($p < 0.01$) reduced in the GD 13 to 17 treatment group. Likewise, significantly ($p < 0.01$) reduced weight gain and viability was observed in GD 13 to 17 litters. In experiment 3, male pups exposed on GD 13 to 17 weighed significantly ($p < 0.05$) less than controls after 15 months. The authors observed that unlike microcystin-LR and anatoxin-a, this study indicates cylindrospermopsin to be an *in utero* developmental toxicant.

7.3.1.6 Chronic Toxicity

No information regarding the chronic toxicity of cylindrospermopsin was located.

7.3.1.7 Carcinogenicity

***In-vivo* Studies**

The tumor initiating activity of cylindrospermopsin was tested in male Swiss mice using O-tetradecanoylphorbol 13-acetate (TPA) as the promoter (Falconer and Humpage, 2001). Mice were administered a gavage dose of saline (27 mice) or 500 mg/kg of a saline extract of freeze-dried *C. raciborskii* cells (strain AWT 205) (34 mice) every other week for three doses. Other groups received a single dose of 1500 mg extract/kg (14 mice) or two doses of 1500 mg extract/kg separated by 2 weeks (17 mice). Most (70%) of the 2 x 1500 mg extract/kg group died within 1 week of the second dose, leaving five survivors for use in the rest of the study. Based on a reported cylindrospermopsin content of 5.5 mg/g extract, the cylindrospermopsin-equivalent doses in the 500 and 1500 mg extract/kg groups were 2.75 and 8.25 mg/kg, respectively. Two weeks after the final dose, the saline and 500 mg extract/kg groups were divided into subgroups

of 13-18 mice that were fed liquid food containing TPA dissolved in DMSO, or food containing DMSO alone, for 24 hours twice weekly for 30 weeks. All of the mice in both 1500 mg extract/kg groups were similarly exposed to TPA-containing liquid food (no 1500 mg/kg mice were exposed to food containing DMSO alone).

Histological examinations of the liver, kidneys, spleen and grossly abnormal organs were performed on all groups at the end of the 30-week promotion period. Neoplastic changes were found in none of the 27 control mice and in a total of 5 cylindrospermopsin-treated mice, a difference that was not statistically significant. There was no pattern to the neoplasic changes, as they occurred in different animals, target organs and treatment groups, as detailed in Table 7-16. The results of the study do not indicate that the cyanobacterial extract was a tumor initiator. However, the study is limited by the design of the dosing regime and by the 30 week observation period.

Table 7-16. Tumor Initiating Activity of *C. raciborskii* Extracts

Oral Treatment (mg extract/kg)	Number of Mice	Histological Finding*
Saline/DMSO	14	No neoplasia observed
Saline/TPA	13	No neoplasia observed
3 x 500/DMSO	18	1 hepatocellular carcinoma 1 lymphoma
3 x 500/TPA	16	No neoplasia observed
1 x 1500/TPA	14	2 hepatocellular dysplastic foci 1 fibroblastic osteosarcoma
2 x 1500/TPA	5	No neoplasia observed

* All findings were in different animals
Source: Falconer and Humpage (2001)

***In-vitro* Studies**

The carcinogenic potential of cylindrospermopsin was assessed *in vitro* via the cell transformation assay (CTA) on Syrian hamster embryo (SHE) cells. This assay is recommended by OECD Guidelines (2007) as an alternative to *in vivo* long term experiment for carcinogenic potential of chemicals (Marie et al., 2010). Purified cylindrospermopsin, supplied by the Australian Water Quality Centre (>98% purity, Adelaide, Australia) was dissolved in water and applied to SHE cells at cylindrospermopsin concentrations of 1 x 10^{-5} to 1 ng/mL (1 x 10^{-8} to 1 x 10^{-3} µg/mL) (first experiment) and 1 x 10^{-7} to 1 x 10^{-3} ng/mL (1 x 10^{-10} to 1 x 10^{-6} µg/mL) (second experiment) for 7 days. Pooled results from both experiments indicate a significant ($p < 0.01$) increase in morphological cell transformation at concentrations ranging from 1 x 10^{-7} to 1 x 10^{-2} ng/mL (1 x 10^{-10} to 1 x 10^{-5} µg/mL). In this study, cylindrospermopsin exhibited transformation at concentrations lower than the ones shown to be genotoxic *in vitro*. Morphological cell transformation occurred at 1 x 10^{-7} ng/mL which indicates a carcinogenicity hazard at very low doses. It is noted that in this study, cylindrospermopsin concentrations >1 x 10^{-2} ng/mL failed to induce cell transformation but were not cytotoxic until 10 ng/mL. This may

be due to a different biotransformation pattern of cylindrospermopsin low doses compared to high doses. It was concluded by the authors that the cell transforming activity indicates carcinogenic potential

In another study, a significant decrease in hepatic P450 of mice treated with cylindrospermopsin *in vivo* was observed compared to control (Terao et al., 1994). At high cylindrospermopsin concentrations, the decrease of P450 enzymes may reduce the production of active metabolites involved in cell transformation.

7.3.2 Other Key Data

7.3.2.1 Mutagenicity and Genotoxicity

A review of the available literature indicates there are no standard mutagenicity studies (Ames Tests) available for cylindrospermopsin. Studies investigating the *in vitro* and *in vivo* genotoxicity (evaluation of DNA damage) from exposure to cylindrospermopsin are few in number and are discussed below.

***In-vitro* Studies**

The genotoxicity of cylindrospermopsin was assessed *in vitro* with two human cell lines (HepaRG and Caco-2) that represent known target organs of cylindrospermopsin (Bazin et al., 2010). In their differentiated state, HepaRG cells express metabolic enzymes at levels comparable to those found in cultured primary human hepatocytes. Therefore, HepaRG are metabolically competent cells derived from a human hepatoma that represent a suitable model to study the genotoxicity of protoxicants in human liver. However, as the major route of human exposure to cylindrospermopsin is likely to be ingestion of contaminated water (i.e., during recreational activities or from drinking), cylindrospermopsin genotoxicity was also investigated in the human colon adenocarcinoma cell line, Caco-2. After differentiation, Caco-2 cells display morphological and biochemical characteristics of human enterocytes. Cylindrospermopsin genotoxicity was assessed using the cytokinesis-block micronucleus assay to assess various cytotoxic and genotoxic outcomes in these cells. The objective of this study was to investigate how changes in phenotype associated with cell differentiation affect toxic response to cylindrospermopsin exposure. In addition, the involvement of CYP metabolism in the cytotoxicity and genotoxicity of cylindrospermopsin was determined by the addition of the CYP3A4 inhibitor ketoconazole.

In the study, cylindrospermopsin (>98% purity, from the Australian Water Quality Center in Adelaide, Australia) was dissolved in physiological saline. Human HepaRG cells (liver-derived) and Caco-2 cells in both differentiated and undifferentiated states were exposed to cylindrospermopsin at concentrations ranging from 0.5 to 2 µg/mL (Caco-2 cells) and 0.04 to 2 µg/mL (HepaRG cells) for 24 hours. Exposure to 1.25–1.5 µg/mL cylindrospermopsin resulted in a significant increase in micronucleated binucleate cells (MNBNC) by approximately three-fold above controls in both differentiated and undifferentiated Caco-2 cells (Table7-15). Above this concentration, the MNBNC frequency reached a plateau. Similarly, in differentiated HepaRG cells, MNBNC increased to a maximum of 1.8-fold over controls at 0.06 µg/mL and

then leveled-off above this concentration. In contrast, cytotoxicity increased through the concentration range in all celltypes. It is likely that MNBNC induction is limited above a certain concentration due to a change in cellular fate from genotoxicity to cytotoxicity. This induction of genotoxicity is in accordance with previous studies showing that cylindrospermopsin induced an increase in micronucleated cells in human lymphoblastoid WIL2-NS cells (Humpage et al., 2000b) as well as DNA breaks both *in vitro* in mouse primary hepatocytes (Humpage et al., 2005) and *in vivo* in Balb/c mouse liver cells (Shen et al., 2002; Straser et al., 2011).

Lankoff et al. (2007) examined the carcinogenic potential of cylindrospermopsin *in vitro* through the formation of chromosomal aberration in Chinese hamster ovary (CHO)-K1 cells. Cylindrospermopsin isolated from two cultures of *C. raciborskii*, AWT 205 (Australian Water Technology center) and Thai (fish pond in Thailand), was prepared in solution and CHO-K1 cells were exposed to 0, 0.05, 0.1, 0.2, 0.5, 1, and 2 µg/mL with and without metabolic activation (S9) for 3, 16, and 21 hours. Results indicate no significant influence on the frequency of chromosome aberrations in cells treated with cylindrospermopsin with or without S9 compared to control groups. The study showed that neither cylindrospermopsin nor the S9 fraction-induced metabolites were clastogenic in CHO-K1 cells. However, significant ($p < 0.05$) decreases in frequencies of mitotic indices after various exposure durations were observed at 0.1 µg/mL and above exposure concentrations. Furthermore, significant ($p < 0.05$) increases in frequencies of apoptotic cells (1 µg/mL and above) and necrotic cells (0.5 µg/mL and above) after 21 hours were observed compared to the controls in a dose and time-dependent manner. Moreover, the presence of metabolic activation influences a susceptibility to necrotic cell death but not an apoptotic one.

To confirm that cylindrospermopsin metabolism is necessary for the manifestation of genotoxicity and to help characterize CYP involvement in activation, the micronucleus assay was also conducted with a CYP inhibitor. CYP3A4 is the major CYP form in the human small intestine, metabolizing the greatest number of drugs and a very large number of other xenobiotics (Pelkonen et al., 2008). Cells were treated with ketoconazole, which is widely known to inhibit CYP3A4. Results indicate ketoconazole strongly protected undifferentiated Caco-2 cells from the induction of (micronuclei) MN induced by cylindrospermopsin suggesting that a CYP-mediated metabolite is involved in the genotoxic effect at noncytotoxic concentrations in the Caco-2 cell model. This finding is in agreement with Humpage et al. (2005) who demonstrated that omeprazole, a CYP3A4 inhibitor less specific than ketoconazole, was effective in protecting mouse primary hepatocytes from cylindrospermopsin-induced genotoxicity. These results are also in accordance with Fessard and Bernard (2003) and Lankoff et al. (2007) who observed that cylindrospermopsin does not react directly with DNA in metabolically-incompetent CHO K1 cells (Table7-17).

In another study by Humpage et al. (2000b), purified cylindrospermopsin caused an increase in the frequency of micronuclei in the human lymphoblastoid cell line, WIL2-NS. WIL2-NS cells were exposed to 1-10 µg/mL cylindrospermopsin for 24 hours to evaluate micronucleus frequency and cellular ploidy. Cylindrospermopsin caused a dose dependent increase in the incidence of MN in BNCs with 10 µg/mL treatment causing an 8 fold increase in MN/1000BNCs over the control. Cylindrospermopsin also produced multimicronucleated cells indicating chromosomal damage, but the mechanism was unclear. An increase in presence of

centromeres was observed in MNBNCs suggesting cylindrospermopsin might be a spindle poison or cause damage to the centromere/kinetochore function. Thus, two mechanisms were suggested for causing the cytogenetic damage: one at the level of DNA to induce strand breaks and the other at the level of kinetochore/spindle function to induce loss of whole chromosomes (Humpage et al., 2000b; Shen et al., 2002).

Fessard and Bernard (2003) examined the genotoxic potential of cylindrospermopsin in (CHO) K1 using the comet assay. Purified cylindrospermopsin caused cell growth inhibition and altered cell morphology, but no apoptosis or DNA strand breaks after 24 h of treatment with cylindrospermopsin at concentrations of 0.5 and 1.0 µg/mL.

Increases in micronucleated cells, micronuclei, nuclear bridges, and nuclear buds were observed in human peripheral blood lymphocytes after a 4 or 24 hour incubation of 0.5 µg/mL cylindrospermopsin or after a 24-hour incubation at a concentration of 0.1 µg/mL. This was accompanied by a decrease in nuclear division index at 0.5 µg/mL after 24 hours (Zegura et al., 2011b). In addition, significant increases in micronuclei, micronucleated cells, and nuclear buds were observed at concentrations ≥0.05 µg/mL. A significant decrease in nuclear division index was observed in HepG2 cells incubated with cylindrospermopsin for 24 hours at a concentration of 0.5 µg/mL (Straser et al.,2011).

The results of the *in vitro* genotoxicity are summarized in Table 7-17 below.

Table 7-17. Genotoxicity of Cylindrospermopsin *In vitro*

Species (test system)	End-point	Results	Reference
Human cell lines (HepaRG and Caco-2)	DNA damage	Exposure to 1.25–1.5 µg/mL resulted in a significant increase in MNBNC in both HepaRG and Caco-2 cells	Bazin et al., 2010
Human lymphoblastoid WIL2-NS cells	DNA damage	Exposure to 1,3,6 and 10 µg/mL increased frequency of MN in WIL2-NS cells	Humpage et al., 2000b
Chinese Hamster Ovary-K1 cells	DNA damage	Comet assay showed altered cell growth and morphology but no interaction with DNA at 0.5 and 1.0 µg/mL	Fessard et al., 2003
Chinese Hamster Ovary-K1 cells	DNA damage	Chromosome aberration not observed in CHO-K1 cells; apoptotic cells (1 µg/mL and above) and necrotic cells (0.5 µg/mL and above) observed	Lankoff et al., 2007
Hepatocytes from Male Albino Swiss Mouse	DNA damage	Comet assay showed concentration dependent increase in comet tail length, area, and moment in cells at 0.05 µM - 0.5 µM	Humpage et al., 2005

***In-vivo* Studies**

DNA strand breakage was observed in the liver of Balb/c mice following a single 0.2 mg/kg i.p. dose of purified cylindrospermopsin (Shen et al., 2002). Significant ($p < 0.001$ to 0.05) DNA strand breakage (characterized by decreased median molecular length fragments) was observed in exposed mouse livers compared to controls from animals sacrificed at 6, 12, 24, 48 and 72 hours after dosing.

Covalent binding of cylindrospermopsin or a metabolite to DNA was detected in the liver of Quackenbush mice given a single i.p. injection of a cell-free extract of *C. raciborskii* (dose levels not reported). A single adduct spot was observed in each case (Shaw et al., 2000). Based on structural characteristics of cylindrospermopsin (its nucleoside structure and potentially reactive guanidine and sulfate groups), it has been speculated that cylindrospermopsin may exert its toxic effects via pathways that include reactions with DNA and/or RNA (see Humpage et al., 2000b; Shen et al., 2002). Covalent binding between DNA and cylindrospermopsin, or a metabolite, occurred in mouse liver *in vivo* (Shaw et al., 2000). The presence of adducts was investigated through DNA hydrolysis into individual nucleotides, labeling of the nucleotides using ^{32}P-ATP, separation of nucleotides using two-dimensional thin layer chromatography and visualization by autoradiography. DNA-base adducts were detected but not identified.

7.3.2.2 Immunotoxicity

No information was located regarding effects of cylindrospermopsin on immune function, although immune system tissues appear to be a target of short-term, high-level exposures. Massive necrosis of lymphocytes occurred in the cortical layer of the thymus of male ICR mice given a single 0.2 mg/kg i.p. dose of cylindrospermopsin purified (purity not reported) from cultured *U. natans* cells (Terao et al., 1994). Effects observed in MF1 mice administered a single gavage dose of a suspension of freeze-dried *C. raciborskii* cells, in the lethal dose range of 4.4-8.3 mg/kg, included atrophy in lymphoid tissue of the spleen (follicular lymphocyte loss due to lymphophagocytosis) and thymus (degeneration and necrosis of cortical lymphocytes) (Seawright et al., 1999). Lympho-phagocytosis was observed in the spleen of Quackenbush mice exposed to a cell-free extract of freeze-dried and sonicated *C. raciborskii* cells by gavage at a nonlethal dose level of 0.05 mg/kg-day for 14 days (Shaw et al., 2000, 2001). These effects were considered to be normal responses of the immune system to the stress of severe intoxication.

7.3.2.3 Physiological or Mechanistic Studies

7.3.2.3.1 Noncancer Effects

Liver

The liver has been widely regarded as the primary target of cylindrospermopsin toxicity, and consequently, most mechanistic studies have assessed hepatic endpoints. The specific mechanism for the liver toxicity is not clearly understood, although it has generally been considered to involve cylindrospermopsin-induced inhibition of protein synthesis. Recently, cytotoxic mechanisms have also been implied in liver toxicity.

Cylindrospermopsin was shown to be a potent inhibitor of protein synthesis in an *in vitro* rabbit reticulocyte globin synthesis assay (Terao et al., 1994). Ultrastructural liver changes in mice treated with a single 0.2 mg/kg i.p. dose of purified cylindrospermopsin had features in common with those dosed with the protein synthesis inhibitor cycloheximide, particularly detachment of ribosomes from the rough endoplasmic reticulum, suggesting that protein synthesis inhibition plays a role in cylindrospermopsin hepatotoxicity *in vivo* (Terao et al., 1994). Cycloheximide exerts its effect by interfering with the translocation step in protein synthesis (movement of two tRNA molecules and mRNA in relation to the ribosome) thus blocking translational elongation. Since cycloheximide is an effective inhibitor of protein biosynthesis in eukaryotes only, it may be used to distinguish between proteins translated in the mitochondria and proteins translated in the cytosol. However, unlike the liver in the cycloheximide-dosed mice, the liver of those treated with cylindrospermopsin showed membrane proliferation, fat droplet accumulation and reduced amount of total P450 in microsomes, indicating that mechanisms other than protein synthesis inhibition must also contribute to cylindrospermopsin toxicity.

Cylindrospermopsin-induced depletion of mouse hepatic glutathione was demonstrated *in vivo* (Norris et al., 2002), although the study authors did not consider the effect to be of sufficient magnitude to represent the primary mechanism of cylindrospermopsin toxicity. Cylindrospermopsin caused decreased glutathione levels, as well as decreased synthesis of glutathione and protein, in cultured rat hepatocytes (Runnegar et al., 1994b, 1995c, 2002). Inhibition of glutathione synthesis was the predominant mechanism for the reduction in glutathione; other mechanisms, including increased utilization of glutathione, increased formation of oxidized glutathione, increased glutathione efflux, decreased glutathione precursor availability and decreased cellular ATP were effectively ruled out (Runnegar et al., 1995c).

Glutathione depletion occurred at non-toxic cylindrospermopsin concentrations and preceded the onset of observable toxicity at higher concentrations (Runnegar et al., 1994b). Pretreatment with the CYP450 inhibitor, α-naphthoflavone, partially protected against cytotoxicity and cellular glutathione depletion, indicating involvement of the CYP450 enzyme system in cylindrospermopsin metabolism and that one or more metabolites might be more active than the parent compound in inhibiting glutathione synthesis (Runnegar et al., 1995c). *In vitro* studies in mouse hepatocytes provided no indication that reductions in glutathione levels by cylindrospermopsin led to increased levels of reactive oxygen species (ROS) (Humpage et al., 2005).

Cylindrospermopsin induced time- and concentration-dependent toxicity and inhibition of protein synthesis in hepatocytes isolated from male Swiss mice (Froscio et al., 2003). The broad-spectrum CYP450 inhibitors proadifen (SKF525A) and ketoconazole diminished the induction of cytotoxicity by cylindrospermopsin, but did not diminish the inhibition of protein synthesis. These findings suggest that the cytotoxic effects of cylindrospermopsin might be linked more to CYP450-mediated bioactivation than to inhibition of protein synthesis by the parent compound. Similarly, pretreatment of male Quackenbush mice with the broad-spectrum CYP450 inhibitor piperonyl butoxide protected against the acute lethality of cylindrospermopsin (Norris et al., 2002).

In a study using inhibitors of specific CYP450 isoforms, furafylline (CYP1A2) and omeprazole (CYP3A4 and CYP2C19) protected against cylindrospermopsin cytotoxicity in an *in vitro* mouse hepatocyte system. However, inhibitors of CYPs 2A6, 2D6 and 2E1 were not found to be cytoprotective (Humpage et al., 2005). Additional support for the involvement of CYP450 in the hepatotoxicity of cylindrospermopsin is the finding that liver histopathology is mainly induced in the region (periacinar) where CYP450-catalyzed xenobiotic metabolism occurs (Shaw et al., 2000, 2001).

In a study by Shen (2003), cylindrospermopsin-induced up-regulation of the tissue transglutaminase (tTGase) gene in liver of Balb/c mice following i.p. injection of a single 100 µg/kg dose of cylindrospermopsin. tTGase is a unique member of the TGase (EC 2.3.2.13) family that catalyzes the post-translational modification of proteins via Ca2+-dependent cross-linking reactions (Shen et al., 2003). The up-regulation of tTGase can lead to liver injury (Grenard et al., 2001; Mirza et al., 1997), and has been implicated in diverse biological processes, such as induction of apoptosis (Zhang et al., 1995), differentiation (Shen et al., 2003; Fesus et al., 1987), and morphological changes of cells (Shen et al., 2003; Akimov and Belkin, 2001).

Kidney

The kidney was the most sensitive target in male mice that were exposed to cylindrospermopsin by drinking water and daily gavage for 11 weeks (Humpage and Falconer, 2003). Renal effects in the mice included increased relative kidney weight at >60 µg/kg-day, decreased urinary protein at >120 µg/kg-day and decreased urine specific gravity and proximal renal tubular lesions at 240 µg/kg-day. The noted decrease in urinary protein excretion reflects a specific effect of cylindrospermopsin on protein synthesis, as well as the possibility that the cyanotoxin impacts a functional change in the nephron. Effects on the kidney and urine protein levels observed in male mice in both drinking water and gavage studies were observed; however, the biological relevance needs further investigation. Information supporting the hypothesis that the decrease in urinary protein excretion reflects a specific effect of cylindrospermopsin on protein synthesis, as well as the possibility that it reflects a functional change in the nephron, is discussed below. Also discussed is evidence suggesting a dose-severity progression of kidney effects.

Potential mechanisms for a decrease in urinary protein include a decrease in glomerular filtration (i.e., filtered load) of protein, an increase in resorption of filtered protein and a decrease in secretion of nephrogenic protein. A decrease in glomerular filtration of protein (e.g., µg protein/day) could result from a decrease in serum protein concentration or a decrease in GFR (mL/day). The predominant serum protein in urine of healthy animals (e.g., mice, rats and humans) is albumin (~50% of serum proteins in urine). In the Humpage and Falconer (2003) study, serum albumin concentration increased in mice exposed to cylindrospermopsin, and serum creatinine (a marker of GFR) apparently was unchanged; it was measured but not discussed in the results. Therefore, it is unlikely that glomerular filtration of serum proteins decreased in response to cylindrospermopsin (if a change occurred, it is likely to have been an increase in the rate of filtration of albumin). Furthermore, serum proteins normally account for approximately 15% of total urinary protein (Pesce and First, 1979). The decrease in urinary excretion of protein

observed in Humpage and Falconer (2003) was substantially larger than this (~50%), indicating that the decrease in urinary protein cannot derive solely from a decrease in excretion (i.e., glomerular filtration) of serum proteins.

No information is presented in Humpage and Falconer (2003) that would allow an assessment of tubular resorption of filtered protein (e.g., plasma-to-urine clearance of protein, excretion of low-molecular weight proteins such as $\beta_{2\mu}$ globulin or retinal binding protein).

In healthy mammals, the dominant protein in urine (~50%) is the nephrogenic Tamm-Horsfall protein (THP, uromucoid) (Bachmann et al., 1991, 2005). In the absence of a decrease in filtration or increased resorption of filtered serum protein, the substantial decrease in urinary protein (i.e., ~50%) observed by Humpage and Falconer (2003) would almost certainly have to involve decreased excretion of THP, since it is the predominant protein in urine. Although there are numerous possible mechanisms for an acute change in THP excretion (Bachman et al., 1991), long-term maintenance of lower (i.e., steady-state) rate of urinary excretion of THP requires a decreased rate of synthesis of THP (Bachman et al., 1991, 2005; Schoel and Pfleiderer, 1987).

THP is synthesized exclusively in the thick ascending limb of the loop of Henle (TAL); therefore, a sustained change in THP excretion is likely to reflect a functional change in this region of the nephron. Increases and decreases in THP have been observed in various kidney diseases, and in association with experimental treatments that induce hypertrophy of the TAL, including increased dietary protein (Bachmann et al., 1991). Depletion of THP from the kidney may, in itself, be adverse. Mice deficient in THP (i.e., THP knockout mice) display impaired urine concentrating ability, up-regulation of distal nephron electrolyte transport proteins and increased susceptibility to urinary tract infections (Bachmann et al., 2005; Bates et al., 2004). The decrease in urine specific gravity in animals exposed to cylindrospermopsin in the Humpage and Falconer (2003) study may be indicative of impaired urine concentrating ability and, possibly, related to impaired function of the TAL (i.e., impairment of transport activity in this region of the nephron impairs urine concentrating ability) and/or decreased synthesis of THP.

Additional kidney effects in the Humpage and Falconer (2003) mouse study included proximal renal tubular damage (type and severity of lesions not reported) at the high dose. Clinical effects in the Palm Island incident, in which humans apparently ingested drinking water containing elevated levels of cylindrospermopsin, included renal damage as indicated by loss of water, electrolytes, proteins, ketones and carbohydrates (Blyth, 1980; Griffiths and Saker, 2003). Proteinuria would be expected with proximal tubular damage, as this is the site of resorption of filtered protein. Proteinuria was not observed by Humpage and Falconer (2003), but information on the type and severity of the tubular damage was not reported. Proteinuria did occur in the humans, although other mechanisms could have caused it (e.g., glomerular injury will produce high molecular weight proteinuria). The evidence for proximal tubular damage and functional impairment (e.g., proteinuria, glucosuria) together strengthen the argument that the kidney is a target of cylindrospermopsin and, when considered with decreased protein excretion at lower doses, suggests a dose-severity progression.

7.3.2.3.2 Cancer Effects

No information regarding physiological or mechanistic cancer studies of cylindrospermopsin were identified in the reference materials.

7.3.2.3.3 Interactions with Other Chemicals

No studies of mixtures of cylindrospermopsin with other chemicals were identified. The Caruara outbreak involved exposure of patients at a renal dialysis clinic in Caruaru, Brazil to a mixture of microcystin and cylindrospermopsin. However, the data do not reveal any quantitative information on the toxicity of the mixture compared to its individual components.

7.3.2.4 Structure Activity Relationship

Natural cylindrospermopsin, synthetic (racemic) cylindrospermopsin and selected synthetically-produced cylindrospermopsin structural analogues were assessed for effects on protein synthesis in both the rabbit reticulocyte lysate system and cultured rat hepatocytes (Runnegar et al., 2002). No significant differences were observed in levels of protein synthesis inhibition elicited by natural cylindrospermopsin versus its diol analogue, indicating that the sulfate group might not be a necessary component of cylindrospermopsin-induced protein synthesis inhibition. Additionally, the orientation of the hydroxyl group at C7 in the carbon bridge does not appear to be important, since the C7 epimer of cylindrospermopsin and its corresponding diol exhibited protein synthesis inhibition similar to that elicited by synthetic (racemic) cylindrospermopsin. The cyclopentyl ring and the methyl and hydroxyl groups on the adjacent hexyl ring may be important structural features, because the analogue lacking these features was 500 to 1000-fold less effective in the inhibition of protein synthesis.

The uracil portion of cylindrospermopsin appears to play an important role in cylindrospermopsin toxicity. Banker et al. (2000) found that the acute lethality of cylindrospermopsin to mice was eliminated by chlorination or partial cleavage of the uracil moiety (resulting in 5-chloro-cylindrospermopsin and cylindrospermic acid, respectively), as shown by a 5-day i.p. LD_{50} value of 0.2 mg/kg for cylindrospermopsin and 10-day i.p. LD_{50} values of >10 mg/kg for 5-chloro-cylindrospermopsin and >10 mg/kg for cylindrospermic acid.

Deoxycylindrospermopsin, an analogue of cylindrospermopsin isolated and purified from *C. raciborskii*, was tested for toxicity in male white Quackenbush mice treated by i.p. injection (Norris et al., 1999). Deoxycylindrospermopsin did not appear to be toxic during 5 days following administration of a 0.8 mg/kg dose, whereas Ohtani et al. (1992) reported a five- to six-day i.p. LD_{50} value of 0.2 mg/kg for cylindrospermopsin in male CD3 mice. Although this comparison suggests that deoxycylindrospermopsin is significantly less toxic than cylindrospermopsin, differences in study designs (e.g., the use of different strains of mice) could have contributed to the difference in toxicity.

7.3.3 Hazard Characterization

7.3.3.1 Synthesis and Evaluation of Major Noncancer Effects

Information on the health effects of cylindrospermopsin in humans is limited to observations on the Australian Palm Island poisoning incident that involved acute and/or short-term drinking water exposure to *C. raciborskii*, a non-infectious cyanobacterium (Blyth, 1980; Griffiths and Saker, 2003). The clinical picture of the illness is well-defined and includes fever, headache, vomiting, bloody diarrhea, hepatomegaly and kidney damage with loss of water, electrolytes and protein, but no data are available on exposure levels of cylindrospermopsin that induced these effects. Furthermore, it is known that the lake (source of drinking water) was treated with copper sulfate to control harmful algal blooms before the observed illness thus the presence of elevated copper concentrations in the drinking water might have been a contributor to the symptoms observed.

The preponderance of information on noncancer effects of cylindrospermopsin in animals is available from oral and i.p. administration studies in mice that were exposed to purified compound or extracts of *C. raciborskii* cells. It is noted that toxicity studies with extracts do not contain purified cylindrospermopsin thus the potential exists for the presence of other cyanotoxins in the dosing solutions. Based on available studies, the liver and kidneys appear to be targets of cylindrospermopsin toxicity. The mechanism for liver toxicity is incompletely characterized, but involves inhibition of protein synthesis (Froscio et al., 2003; Terao et al., 1994). Available evidence indicates that the protein synthesis inhibition is not decreased by broad-spectrum CYP450 inhibitors, suggesting that it is mediated by the parent compound (Froscio et al., 2003). Hepatocytotoxicity that occurs at higher levels of exposure to cylindrospermopsin appears to be CYP450-dependent, indicating the involvement of metabolites and mechanisms other than protein synthesis inhibition (Froscio et al., 2003; Humpage et al., 2005; Norris et al., 2002).

Studies specifically investigating the inhibition of protein synthesis in the kidneys are not available, although the results of the 11-week oral toxicity study in mice (Humpage and Falconer, 2003) are consistent with an inhibition of protein synthesis. Effects in this mice study included decreased urinary protein and, at a higher dose, proximal renal tubular lesions. Proximal renal tubular damage in mice (Humpage and Falconer, 2003), as well as the clinical findings of renal insufficiency in the Palm Island human poisoning incident (Blyth, 1980; Griffiths and Saker, 2003), suggest that cytotoxic mechanisms may predominate in the kidney. As hypothesized by the authors, the decrease in urinary protein could stimulate kidney growth and could also reflect whether the renal effects are species specific.

Short-term animal dosing experiments (Shaw et al., 2001) suggested a LOAEL of 150 $\mu g\ kg^{-1}\ day^{-1}$ based on lipid infiltration of the liver and lymphagocytosis in the spleen as toxicity end points (Table 7-18). In a more detailed investigation of the *in vivo* effects of subchronic oral exposure, Humpage and Falconer (2003) gavaged groups of 10 mice with 0, 30, 60, 120 or 240 µg/kg-d of purified cylindrospermopsin (extract of freeze-dried *C. raciborskii* cells (strain AWT 205)), in water for 11 weeks. A number of parameters were measured, but kidney to body weight ratios were increased at the lowest dose. Additional renal effects observed were decreased

urinary protein at >120 µg/kg-day and increased relative kidney weight at >60 µg/kg-day, both are potential indicators of suppressed protein synthesis. As hypothesized by the authors, the decrease in urinary protein is consistent with decreased availability of protein and the increase in kidney weight may reflect a compensatory hyperplasia, such that the kidney, as a protein-synthesizing organ, is stimulated to grow in an attempt to maintain homeostasis in response to a chemically-induced decrease in protein synthesis. The noted decrease in urinary protein excretion reflects a specific effect of cylindrospermopsin on protein synthesis, as well as the possibility that this cyanotoxin impacts a functional change in the nephron. Based on renal

Table 7-18. Summary of Noncancer Results in Animal Studies of Oral Exposure to Cylindrospermopsin

Species	Sex	Average Daily Dose (µg/kg-day)	Exposure	NOAEL (µg/kg-day)	LOAEL (µg/kg-day)	Responses	Comments	Reference
Acute Exposure								
No suitable studies available								
Short-Term Exposure								
Mouse	NR	0.05, 0.15,0.3 (gavage with purified cell extract of *C. raciborskii*)	14 days	50	150	Lipid infiltration in liver.	Low confidence in NOAEL and LOAEL. A full report of this study has not been published; limited information on experimental design and results.	Shaw et al., 2000, 2001
Sub-chronic Exposure								
Mouse	M	0, 0.03, 0.06, 0.12, 0.24 (drinking water with purified extract of *C. raciborskii* (AWT 205)	11 weeks	30	60	Increased relative kidney weight; and decreased urinary protein at >120 µg/kg-day.	Well-designed study with endpoints that included food and water consumption, body weight, clinical signs, hematology, serum chemistry, urinalysis, organ weights (eight organs) and histology (comprehensive). Ten mice/level (six in high dose group).	Humpage and Falconer, 2003
Mouse	M, F	Daily increase from 10 to 55 µg/kg-day for 42 weeks (drinking water)	42 weeks	ND	20	After 16 weeks, hematocrit increase, anthrocytes present; After 42 weeks increased kidney and liver weight	20 male and 20 female mice/level; spent medium from actively growing *Aphanizomenon ovalisporum* as dosing agent (purity unknown); step-up dose study; uncertain quantification of dose in drinking water	Sukenik et al., 2006

effects, this study identifies a NOAEL and LOAEL of 30 and 60 µg/kg-day for extracts from *C. raciborskii* cells (strain AWT).

In a long-term study by Sukenik et al. (2006), exposure of male and female mice to extract containing cylindrospermopsin in a dose step-up study via drinking water resulted in structural changes in the RBC which were associated with increased hematocrit value as an indicator for adverse changes after 20 weeks of exposure to cylindrospermopsin with increased kidney and liver weights observed after 42 weeks. Humpage and Falconer (2003) failed to see comparable RBC changes in their 13 week mouse subchronic study.

No information was located regarding the chronic toxicity, immunotoxicity, or neurotoxicity of cylindrospermopsin. There is some evidence that cylindrospermopsin can have an impact on postnatal growth from an i.p, study in CD-1 mice (Rogers et al., 2007).

7.3.3.2 Synthesis and Evaluation of Major Carcinogenic Effects

Studies investigating the *in vitro* and *in vivo* genotoxicity (evaluation of DNA damage) from exposure to cylindrospermopsin are few in number. *In vitro* mutagenic and genotoxic cell assays with cylindrospermopsin have shown varied results with some indications of potential damage to DNA. The human hepatocytic and enterocytic models consisting of HepaRG and Caco-2 cells showed increased MNBNC and micronucleated cells were observed in a study with human lymphoblastoid WIL2-NS cells. DNA breaks have been observed in primary hepatocytes by the comet assay indicating that DNA strand breakage could be a mechanism for cylindrospermopsin-induced cytogenetic damage. Following i.p. exposure, DNA strand breakage was observed in the liver of Balb/c mice and covalent binding between DNA and cylindrospermopsin, or a metabolite, occurred in Quackenbush mouse liver. However, these data are limited and there has been no long term bioassay of purified cylindrospermopsin with known doses. The limited study by Falconer and Humpage (2001) on initiation with TPA promotion did not support classification of cylindrospermopsin as a tumor initiator.

7.3.3.3 Mode of Action and Implications in Cancer Assessment

There is minimal information available to inform a cancer mode of action hypothesis for cylindrospermopsin lacking any bioassay data. The study by Falconer and Humpage (2001) noted only one tumor and two areas of dysplastic foci in a study of two doses of *C. raciborskii* extracts or three doses of freeze dried cells combined in treatment with TPA as a promter. However, only 14 or 15 animals were tested and only 5 of the group receiving the second extract dose survived for more than one week. Genotoxic studies indicate a potential for cylindrospermopsin to affect DNA in mouse liver however, these data are limited.

7.3.3.4 Weight of Evidence Evaluation for Carcinogenicity

In accordance with the Guidelines for Carcinogen Risk Assessment (U.S. EPA, 2005a), the weight of evidence descriptor for the carcinogenic hazard potential of cylindrospermopsin is

"Inadequate Information to Assess Carcinogenic Potential." No data are available to support an assessment of carcinogenic potential.

7.3.3.5 Potentially Sensitive Populations

There is no information on possible age or gender differences in the disposition of, or response to, cylindrospermopsin. No data were located regarding populations that might be unusually susceptible to cylindrospermopsin. It is conceivable that individuals with liver and/or kidney disease might be more susceptible than the general population because of compromised detoxification mechanisms in the liver and impaired excretory mechanisms in the kidney. The case study data from the Palm Island incident and the developmental toxicity data indicate that children and the fetus might be more sensitive than adults to early life exposure to cylindrospermopsin. However, the supporting data are weak.

8.0 DOSE-RESPONSE ASSESSMENT

8.1 Microcystins

8.1.1 Dose-Response for Noncancer Effects

Human Data

Human data on the oral toxicity of MC-LR are limited by lack of quantitative information and by potential co-exposure to other cyanobacterial toxins and microorganisms. Anecdotal reports indicate that, in humans, exposure to cyanobacterial blooms (including microcystin-producing genera) can result in neurological, gastrointestinal and dermatological symptoms, such as headache; muscle weakness; eye, ear and throat irritation; nausea; stomach pain; diarrhea; blistering around the mouth; and hay-fever like symptoms (Dillenberg and Dehnel, 1960; Billings, 1981; Turner et al., 1990; Teixeira et al., 1993; el Saadi and Cameron, 1993). Effects were reported in persons exposed via recreational contact and drinking water. Turner et al. (1990) also reported pneumonia in army recruits exposed to a cyanobacterial bloom. Symptoms occurring after exposure to cyanobacteria cannot be directly attributed to microcystin toxins or other endotoxins; some effects may result from exposure to the cyanobacterial cells themselves, or from exposure to multiple toxins in the bloom.

Animal Data

The toxicological database for microcystins is almost exclusively limited to data on a single congener, MC-LR. Data on the other RR, YR, and LA congeners are limited such that toxicity values cannot be derived for these congers. The database on the oral toxicity of MC-LR is adequate to support the derivation of the RfD (Table 8-1). Animal studies of oral exposures to microcystins focused on toxicity to target organs, mainly liver and testes.

A subchronic study in mice identified a LOAEL of 200 µg/kg/day, which resulted in mild liver lesions including chronic inflammation, hemosiderin deposits and single hepatocyte degeneration , as well as increased ALT and AST in male animals. The NOAEL was identified as a dose of 40 µg/kg/day (Fawell et al., 1999a). No liver or other toxicity was reported in female mice given approximately 3 µg/kg/day of MC-LR in drinking water for 18 months (Ueno et al., 1999). Male mice administered 8 or 16 µg MC-LR/kg/day via the drinking water for up to 270 days showed mild hepatocyte injury, increased relative liver weight, and decreased body weight; the NOAEL was 0.2 µg/kg/day (Zhang et al., 2010; 2012). The Uneo et al. (1999) and Zhang et al. (2010; 2012) provide supporting information for liver toxicity, but do not provide sufficient data for dose-response analyses.

Male mice administered MC-LR via their drinking water at a low concentration, resulting in a dose of 0.79 µg/kg/day, had decreased sperm counts and sperm motility after 3 and 6 months (Chen et al., 2011). At 6 months increased sperm abnormalities, decreased serum testosterone, and increased serum LH levels were also observed. Testes weights, however, were not affected and the NOAEL was 0.25 µg/kg/day. Similar adverse effects on sperm were observed in rats administered 0.5 µg/kg of toxin extract or 10 mg/kg of biomass of *M. aeruginosa* for three

months (Kirpenko et al., 1981). A number of *in vivo* and *in vitro* studies with both pure MC-LR and cellular extracts support the testes as a target organ; these were described in Section 7.1.3.5.

The NOAELs for the target organs of liver (0.2 µg/kg/day; Zhang et al., 2010, 2012) and testes (0.25 µg/kg/day; Chen et al., 2011) were similar for drinking water exposures to male mice for 6-9 months. Lesions in the testes and effects on sperm motility were observed at doses of 0.79 µg/kg/day of purified MC-LR (Chen et al., 2011) and 0.5 µg/kg/day of an extract (Kirpenko et al., 1981).

The studies by Fawell et al. (1999) and Chen et al. (2011) were well conducted, used an adequate number of animals, and identified both a NOAEL and LOAEL. The Fawell et al. (1999) study was a comprehensive toxicological study that included clinical observations, body weight and food consumption data, eye examinations, hematology and serum biochemistry measurements, and microscopic evaluation of tissues (although the testes were not specifically listed). The Chen et al. (2011) study was not a comprehensive toxicology study, but provided toxicological information on reproductive hormones and the testes.

Table 8-1. Summary of Noncancer Results in Repeated Dose Animal Studies of Oral Exposure to Microcystins

Species	Sex	Average Daily Dose (µg/kg-day)	Exposure	NOAEL (µg/kg-day)	LOAEL (µg/kg-day)	Responses	Comments	Reference
Subchronic Exposure								
Mouse	M/F	0, 40, 200, 1000	Daily gavage, 13 weeks	40	200	Minimal/slight chronic inflammation with hemosiderin deposits and single hepatocyte degeneration; increased serum enzymes (ALT and AST)		Fawell et al., 1999a
Chronic Exposure								
Mouse	F	0, 3	Drinking water, 18 months	3	ND	No effects on survival, body weight, hematology, serum biochemistry, organs or histopathology	Minor changes in ALP and cholesterol not considered toxicologically significant	Ueno et al., 1999
Mouse	M	0, 0.2, 8.0, 16	Drinking water, 180 or 270 days	0.2	8	Histopathological changes in the liver, decreased body weight, increased relative liver weight; MMP expression increased in all groups	Changes in MMP expression and protein levels not considered adverse.	Zhang et al., 2010; 2012
Reproductive Toxicity								
Mouse	M	0, 0.25, 0.79, 2.5 (calculated using a mouse body weight of 0.0316 g and 0.0078 L/day)	Drinking water; 3 or 6 months	0.25	0.79	Decreased sperm counts and motility; lesions in the testes; decreased testosterone, increased LH and FSH		Chen et al., 2011
Rat	M/F	0.0005 or 0.5 (extract) 10 mg/kg (biomass)	Oral; 3 months	0.0005	0.5	Histopath lesions in ovaries and testes; changes in estrous cycle; multiple sperm effects	5×10^{-4} or 5×10^{-7} mg/kg extract; or 10 mg/kg biomass	Kirpenko et al., 1981

8.1.1.1 RfD Determination

Quantitative data are available from Chen et al. (2011) as candidates for the point-of-departure (POD) for RfD derivation. The most sensitive endpoints were sperm motility and sperm count which were affected as early as 3 months after initiation of treatment. Severity of effects on these endpoints increased with continued treatment up to 6 months. Other effects at 6 months included increased in sperm abnormalities and changes in hormone levels. The data for sperm motility and count at 6 months (Table 8-2) were modeled using U.S. EPA's Benchmark Dose software (BMDS; version 2.3.1) for continuous datasets. Data were published by Chen et al. (2011) as group mean and standard error. Therefore the standard errors (S.E.) were converted to standard deviations (S.D.), using the equation S.D. = S.E. × √n, prior to data entry into BMDS.

Table 8-2. Key data from mice exposed to Microcystin-LR in the drinking water for 6 months

Endpoint	0 µg/kg/day	0.25 µg/kg/day	0.79 µg/kg/day	2.5 µg/kg/day
Sperm count ($\times 10^6$/mL)	21.5 ± 2.21	19.7 ± 2.85	13.6 ± 3.48	6.6 ± 2.85
Sperm motility (%)	60.6 ± 16.13	46.8 ± 21.19	23.1 ± 10.12	17.4 ± 15.81

From Chen et al., 2011; mean ± S.D.; n=10

In accordance with the U.S. EPA (2012) BMD methodology for continuous endpoints, the default benchmark response (BMR) of one standard deviation change from the control mean was selected in the absence of a biological rationale for using an alternative BMR. All continuous models were used to fit the data for sperm count and motility and goodness-of-fit information (p value and AIC) was used to choose the best model for each data set. For both data sets, an adequate fit was achieved with the exponential (M4) and polynomial models while sperm count data were also fit with the exponential M2,3 model. Results are shown in Table 8-3 and the corresponding graphic output for each model is shown in Figure 8-1.

Table 8-3. Benchmark model predictions for sperm count and motility

Model	p	AIC	BMD	BMDL
Sperm Count				
Exponential				
M2,3	0.3793	128.4221	0.2755	0.2112
M4	0.2657	129.7221	0.2312	0.1590
Polynomial	0.3471	129.3675	0.2413	0.1772
Sperm Motility				
Exponential				
M4	0.4006	267.7402	0.2127	0.1205
Polynomial	0.9411	267.0392	0.2727	0.1942

Data from Chen et al., 2011

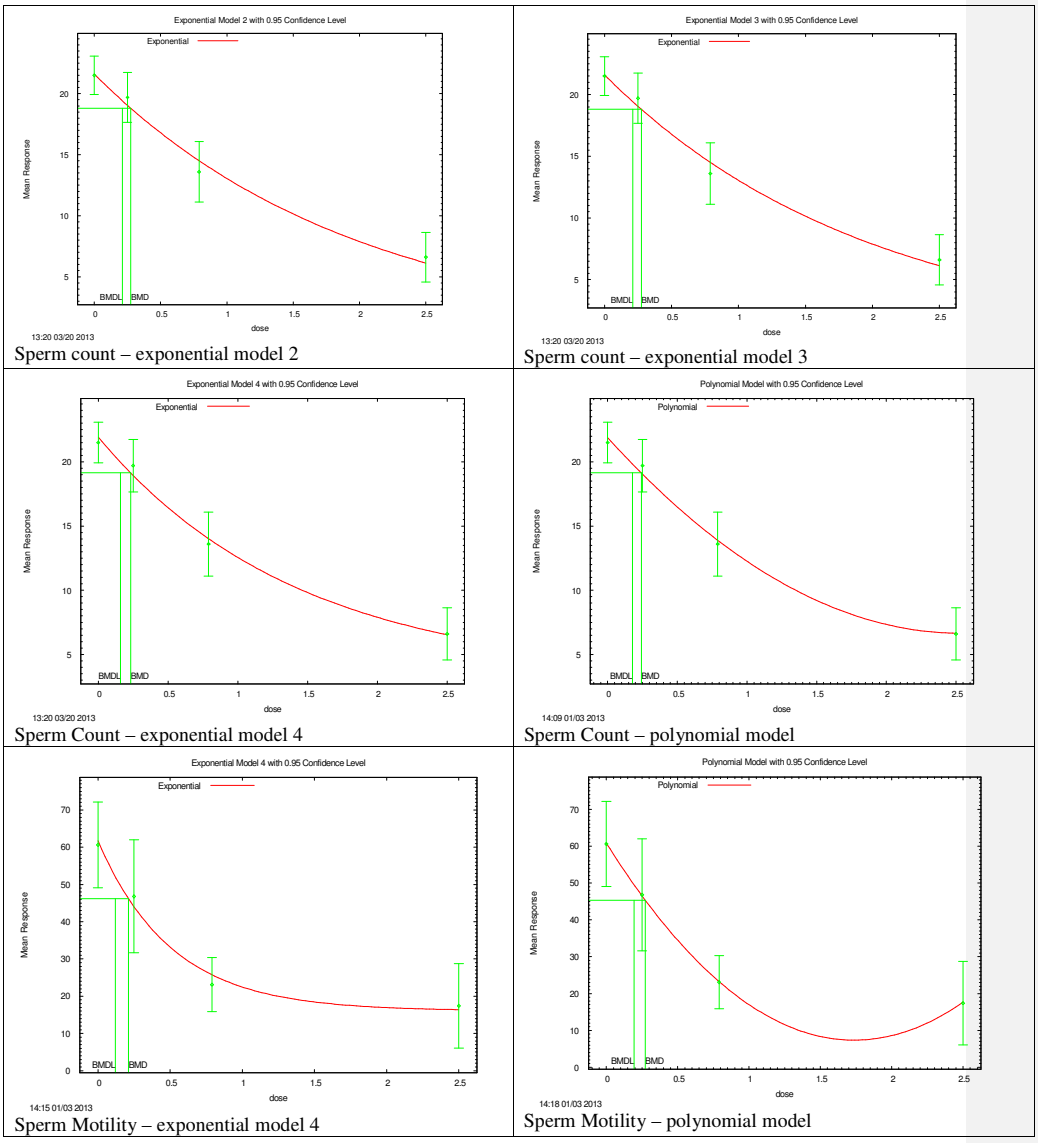

Figure 8-1. BMDS graphic output from selected model runs for Microcystin-LR

As the most sensitive indicator of the effects on the male reproductive system, BMDL of 0.1205 µg/kg/day from the sperm motility data is selected as the POD for the chronic RfD. The

POD originated from a continuous drinking water study, thus no duration adjustment was needed. In lieu of either chemical-specific kinetic models or data to inform the derivation of human equivalent oral exposures, body weight scaling to the ¾ power (i.e., $BW^{3/4}$) was used as a default approach to extrapolate toxicologically equivalent doses from adult laboratory animals to adult humans for the purpose of deriving an oral RfD (U.S. EPA, 2011).

No physiologically-based toxicokinetic modeling information exists for MC-LR. Therefore, the candidate POD for sperm motility is converted to an HED employing a standardized dosimetric adjustment factor (DAF) derived as follows (U.S. EPA, 2011):

$$DAF = (BW_a^{1/4} / BW_h^{1/4})$$
$$DAF = (0.0316)^{1/4} / (70)^{1/4}$$
$$DAF = 0.42/2.89$$
$$DAF = 0.15$$

Where:

DAF = dosimetric adjustment factor
BW_a = animal body weight
BW_h = human body weight

Using a subchronic BW_a of 0.0316 kg for male B6C3F1 mice and a BW_h of 70 kg for humans, the resulting chronic DAF for male mice is 0.15. Applying this DAF to the POD (BMDL of 0.1205 µg/kg/day for decreased sperm motility) identified for the critical effect in mature mice yields a corresponding POD_{HED} as follows:

$$POD_{HED} = \text{POD animal dose (µg/kg/day)} \times DAF$$
$$POD_{HED} = 0.1205 \text{ µg/kg/day} \times 0.15$$
$$POD_{HED} = 0.018 \text{ µg/kg/day or } 1.8 \times 10^{-2} \text{ µg/kg/day}$$

The POD_{HED} of 0.018 µg/kg/day, based on decreased sperm motility, is used to calculate the RfD. At total uncertainty factor (UF) of 300 was applied that included a 10 for intraspecies extrapolation, a 3 for interspecies variability, and a 10 for database insufficiencies. Therefore, the RfD for MC-LR is 6×10^{-5} µg/kg/day.

$$RfD = POD_{HED} \div UF$$
$$= 0.018 \text{ µg/kg/day} \div 300$$
$$= 0.00006 \text{ µg/kg/day or } 6 \times 10^{-5} \text{ µg/kg/day}$$

Uncertainty Factor Application:

UF_H: A default 10-fold for intraspecies differences was used to account for potentially susceptible individuals in the human population. Insufficient information is available on the toxicity of microcystins in exposed humans. Cases of human poisoning have been attributed to ingestion of water containing microcystin-producing cyanobacteria, but no dose-response information is available. No information was found on the degree to which humans of varying gender, age, health status or genetic makeup might vary in the disposition of, or response to, ingested microcystins. Limited data suggests that adult or

aged rodents may be more susceptible than young rodents to the acute toxicity of MC-LR (Ito et al., 1997a).

UF_A: For interspecies uncertainty, a value of 3 ($10^{½}$ = 3.16, rounded to 3) was applied to account for uncertainty in characterizing toxicodynamic differences between mice and humans when an HED was calculated using $BW^{¾}$ scaling, as uncertainty in characterizing toxicokinetic differences was addressed through a standard DAF (U.S. EPA, 2011).

UF_D: A 10-fold factor is used to account for deficiencies in the database. Database deficiencies include the lack of a detailed developmental toxicity study, a neurotoxicity study, a multi-generation reproductive toxicity study and supporting information on systemic toxicity in a second species. The RfD is based on data for MC-LR because few data exist for the other microcystin congeners.

8.1.1.2 RfC Determination

The available data do not provide adequate information for the derivation of the inhalation RfC for microcystins. Two acute inhalation studies were identified in the literature but these are not adequate for derivation of the RfC. One well-conducted study of inhalation exposure to MC-LR was identified. Benson et al. (2005) exposed groups of six male BALB/c mice to monodispersed submicron aerosols of 260-265 µg MC-LR/m^3 via nose-only inhalation for 30, 60 or 120 minutes each day for 7 consecutive days. Histopathological examination revealed treatment-related lesions in the nasal cavity only. The incidence and severity of nasal lesions increased with daily exposure duration. This study used only one exposure concentration, and as such, the data are of limited utility for RfC derivation. Further, extrapolation of the effects from this study for the purpose of deriving the RfC would be associated with substantial uncertainty given the brief exposure time (30-120 minutes/day) and duration (7 days). No subchronic or chronic animal studies evaluating the inhalation route of exposure were available.

8.1.2 Dose-Response for Cancer Effects

Dose-response data regarding the carcinogenicity of microcystins are not available. The few available epidemiological studies on humans that suggest a positive association between liver or colorectal cancers and microcystins have several limitations. No increase in neoplastic liver nodules was found after gavage administration to mice in a chronic bioassay (Ito et al., 1997b). MC-LR has been shown to have a promotional effect in two-stage rat liver bioassays using i.p. administration; however, the relevance of this effect to environmental exposures is uncertain. Mechanistic information provides some support for a possible promotional effect of MC-LR. Applying the Guidelines for Carcinogen Risk Assessment (U.S. EPA, 2005a), there is *inadequate evidence to determine the carcinogenicity* of microcystins.

8.2 Anatoxin-a

8.2.1 Dose-Response for Noncancer Effects

The preponderance of experimental studies of anatoxin-a are *in vitro* and pertain to its mode of neurotoxic action. These studies have established that anatoxin-a is a nicotinic acetylcholine receptor agonist that exerts its effects at both peripheral and central sites in the nervous system. *In vitro* studies also indicate that anatoxin-a can affect non-neuronal cells, causing effects that include apoptosis via production of reactive oxygen species and caspase activation in rat thymocytes and monkey kidney cells, and cytotoxicity without apoptosis in mouse lymphocytes.

The acute *in vivo* neurotoxicity of anatoxin-a in animals is well-documented and characterized by tremors, altered gait, convulsions and death by respiratory paralysis. Little information is available on *in vivo* neurotoxicity at sublethal doses; findings include no effects of gestational i.p. exposure on postnatal neuro-developmental maturation in mice and no effects of acute intravenous exposure on motor activity, coordination, sensory/motor reflexes and other central nervous system responses in mice. Information on the *in vivo* effects of anatoxin-a in orally exposed laboratory animals is available from single-dose lethality assays in mice, 5- and 28-day studies in mice and a developmental toxicity study in mice (Fawell and James, 1994; Fawell et al., 1999b), and a 7-week study in rats (Astrachan and Archer, 1981; Astrachan et al., 1980). However, a limited amount of dose-response data on systemic toxicity and developmental toxicity is available due to limitations in experimental design and reporting, including insufficient numbers of dose levels as well as study endpoints. In particular, the available oral database is limited by a few NOAELs and no LOAELs. The oral toxicity data are insufficient for deriving RfDs.

8.2.2 RfD Determination

The available acute duration oral toxicity data for anatoxin-a are inadequate to support derivation of an acute RfD. Cases of non-lethal human poisonings, manifested mainly as acute gastrointestinal disturbances, have been attributed to ingestion of lake or pond water containing anatoxin-a-producing *Anabaena* sp. (Behm, 2003; Schwimmer and Schwimmer, 1968). Anatoxin-a was implicated in the death of a person who suffered a seizure and heart failure 2 days after swallowing pond water containing *A. flos-aquae* in an algal bloom (Behm, 2003; Carmichael et al., 2004). None of these case reports provide dose information or unequivocally establish anatoxin-a as the causal agent. Acute oral experimental data for anatoxin-a in animals are essentially limited to the results of two lethality assays in mice that determined a single-dose LD_{50} value of 13.3 mg anatoxin-a/kg and identified neurotoxicity as the cause of death (Fitzgeorge et al., 1994; Stevens and Krieger, 1991). Derivation of an acute oral RfD based on the human or animal data is precluded by insufficient information on sensitive endpoints and associated dose-response relationships.

Information on the short-term oral toxicity of anatoxin-a is available from 5- and 28-day systemic toxicity studies in mice and a developmental toxicity study in mice (Fawell and James, 1994; Fawell et al., 1999). The better designed study of these is the 28-day study which tested

groups of 10 mice/sex at dose levels of 0, 0.1, 0.5 and 2.5 mg/kg-day and identified an apparent NOAEL of 2.5 mg/kg-day. The NOAEL is based on the low mortality incidences that showed no dose-response or gender consistency, a lack of clinical signs of acute neurotoxicity prior to death, and a lack of toxicologically significant effects in the surviving animals (comprehensive evaluations were performed that included hematology, clinical chemistry and histology). However, due to the lack of determination of an adverse effect level in the 28-day oral study, data are inadequate to support derivation of a subchronic short-term oral RfD for anatoxin-a.

Information on the subchronic oral toxicity of anatoxin-a is available from a 7-week drinking water study in rats (Astrachan and Archer, 1981; Astrachan et al., 1980). This study identified a NOAEL of 0.5 mg/kg-day but is limited by insufficiencies including only two dose levels, a minimal number of endpoints, and the lack of an adverse effect level. Likewise, the study provides no information on proximity of the 0.5 mg/kg-day NOAEL to the toxicity threshold, due to the lack of a LOAEL or FEL. Thus, insufficient data are available to support derivation of a subchronic oral RfD for anatoxin-a.

8.2.3 RfC Determination

No information is available on the inhalation toxicity of anatoxin-a.

8.2.4 Dose-Response for Cancer Effects

There is no information on carcinogenicity in humans or animals or on possible carcinogenic processes and mode(s) of action for anatoxin-a. Under the Guidelines for Carcinogen Risk Assessment (U.S. EPA, 2005a), the database is inadequate for an assessment of human carcinogenic potential.

8.3 Cylindrospermopsin

8.3.1 Dose-Response for Noncancer Effects

The main source of information on the toxicity of cylindrospermopsin in humans is from qualitative reports of a hepatoenteritis-like illness that is attributed to the acute or short-term consumption of drinking water containing *C. raciborskii*. The clinical picture of the illness includes fever, headache, vomiting, bloody diarrhea, hepatomegaly and kidney damage with loss of water, electrolytes and protein, but no data are available on exposure levels of cylindrospermopsin that induced these effects. From limited oral toxicity studies in animals, cylindrospermopsin is most likely absorbed from the gastrointestinal tract and i.p. toxicokinetic studies indicate that it is mainly distributed to the liver and excreted in the urine as unmetabolized compound. Main targets of cylindrospermopsin toxicity include the liver and kidneys, and possible modes of action include inhibition of protein synthesis, bioactivation to a reactive intermediate and covalent binding of the parent compound or a metabolite to DNA and/or RNA.

The database on oral toxicity of purified cylindrospermopsin in animals is limited by a

small number of studies with appropriate durations. Most of the studies suffer from insufficient reporting of effects considered to be key toxicity endpoints. A single study has been performed assessing the acute oral toxicity of pure cylindrospermopsin, but used only 3 animals per dose and experimental details were poorly documented. Information on short-term oral toxicity is available from 14- and 21-day studies in mice and rats (Shaw et al., 2000, 2001); however, effect levels cannot be assessed due to inadequate reporting of key information on the design and results of these studies. Data on the subchronic oral toxicity of purified cylindrospermopsin from cell extract are available from a comprehensive 11-week study that identified adverse kidney effects in mice based on increased relative kidney weight at >60 µg/kg-day and decreased urinary protein at >120 µg/kg-day (Humpage and Falconer, 2003). The noted decrease in urinary protein excretion reflects an effect of cylindrospermopsin on protein synthesis as well as the possibility that the cyanotoxin impacts a functional change in the nephron. Effects on the kidney and urine protein levels in male mice in both drinking water and gavage studies have been observed; however, the biological relevance is inconclusive due to the lack of systemic or histopathologial effects and needs further investigation.

No information was located regarding the chronic toxicity, neurotoxicity or developmental/reproductive toxicity of cylindrospermopsin following oral exposure.

8.3.2 RfD Determination

The available oral subchronic toxicity data for Cylindrospermopsin are inadequate to support derivation of an acute RfD. The 11-week subchronic study in mice (Humpage and Falconer, 2003) is the only subchronic study of purified cylindrospermopsin but does not provide a suitable basis for RfD derivation. The study indicates an increase in relative kidney weight but is inconclusive due to the lack of histopathologial effects and lack of effects to other toxicity endpoints. No chronic oral studies have been performed and use of the subchronic study for chronic RfD estimation by extrapolation across exposure durations is precluded by the study limitations described above. Derivation of an acute oral RfD based on the human or animal data is precluded by insufficient information on sensitive endpoints and associated dose-response relationships.

8.3.3 RfC Determination

No information is available on the toxicity of inhaled cylindrospermopsin.

8.3.4 Dose-Response for Cancer Effects

There is no dose-response or other information available regarding the carcinogenicity of cylindrospermopsin in reported incidental human exposures or cancer studies in animals. In a few published studies to date, cylindrospermopsin has exhibited genotoxicity in the human hepatocytic and enterocytic models of HepaRG and Caco-2 cells. Likewise, DNA breaks have been observed in mice primary hepatocytes by the comet assay indicating that DNA strand breakage could be a mechanism for cylindrospermopsin-induced cytogenetic damage. However, insufficient data are available to support further assessment of the carcinogenic potential of cylindrospermopsin.

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